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Sex-Bias in Lupus-Prone (NZBXNZW)F1 Mice: The Interplay of GR1+CD11B+ Cells, Testosterone and Genetics

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SEX-BIAS IN LUPUS-PRONE (NZBXNZW)F1 MICE: THE INTERPLAY OF $GR1^{+}CD11B^{+}$ CELLS, TESTOSTERONE AND GENETICS

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DEDICATION

This thesis is dedicated to the loving memory my first ever teacher, my grandfather

Mr. V.P. Trigunait. I wish you were here to cherish this moment.

My mother Kumud Trigunait, you are the rock of our family and the source of never ending inspiration and love.

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SEX-BIAS IN LUPUS-PRONE (NZBxNZW)F1 MICE: THE INTERPLAY OF GR1+CD11b+ CELLS, TESTOSTERONE AND GENETICS

ABHISHEK TRIGUNAITE

ABSTRACT

Systemic Lupus Erythematosus (SLE) is a prototypic systemic autoimmune disorder predominantly (9:1) targeting females. Currently, there is no cure for SLE and also there is a lack of disease predicting biomarkers. The major aim of my research is to determine the cellular basis of the sex bias in SLE and subsequently facilitate the development of better therapeutics and biomarkers for the disease. In (NZBxNZW) F1 mice a similar female bias is observed as in humans and testosterone is shown to be protective against lupus-like disease development, but the mechanism is not well understood.

We found higher levels of $Gr1^{hi}CD11b^{+}$ myeloid cells in (NZBxNZW) F1 male mice as compared to females. Interestingly, $Gr1^{hi}CD11b^{+}$, as well as $Gr1^{low}CD11b^{+}$, cells have been characterized as granulocytic and monocytic *myeloid derived suppressor cells (MDSCs),* respectively. Hence, we hypothesized that - ***$Gr1^{hi}CD11b^{+}$ cells suppress B cell activation and differentiation and protect against lupus-like disease.***

We report here that $Gr1^{hi}CD11b^{+}$ cells from pre-pubertal male and female BWF1 significantly suppress *in vitro* cytokine-mediated B cell differentiation into plasma cells. *In vivo* depletion of $Gr1^{+}$ cells leads to significantly elevated levels of ANA in male

(NZBxNZW)F1 mice. Additionally, $Gr1^{hi}CD11b^{+}$ cells are regulated by male sex hormone.

Our findings also show that ***Gr1+ cells suppress antibody response to antigen challenge in (NZB x NZW) F1 male mice through inhibition of T follicular helper (T_{FH}) cells and germinal center formation.*** The identification of testosterone dependent immune-suppressive cell population in lupus-prone mice can, in part, explain the protective role of testosterone in lupus-like disease development.

We also examined the hormone independent contribution of male versus female immune system in development of lupus like disease in (NZBxNZW)F1 mice. The results of these studies indicate that the intrinsic auto-immune capabilities of the female (NZBxNZW)F1 hematopoietic cells can override the protective effect of testosterone.

Further studies are required to identify the correlation between immune system development and sex hormones with specific emphasis on the identification of molecular targets that may better serve as therapeutic and preventive tools for management of SLE and provide a better quality of life for people suffering from this disease.

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LIST OF ABBREVIATIONS

Ab	Antibody
AIRE	Autoimmune regulator
ANA	Anti-nuclear antibody
APRIL	A proliferation induced ligand
BAFF	B cell activating factor
BCR	B cell receptor
BM	Bone marrow
B_{regs}	Regulatory B cells
CMP	Common Myeloid Progenitor
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein - Barr virus
FL	Fetal liver
HC	Hematopoietic cell
HSC	Hematopoietic stem cell
IC	Immune complex
IFN	Interferon
iNOS	Inducible nitric oxide
mAb	Monoclonal antibody
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
NSAIDS	Nonsteroidal anti-inflammatory drugs
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
Rag	Recombination activating genes
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
TACI	Transmembrane activator and calcium-modulator and cyclophilin-ligand interactor

TCR	T cell receptor
T_{FH}	T follicular helper cells
TGF	Tumor growth factor
T_H	Helper T cell
TLR	Toll-like receptor
TNF	Tumor necrotic factor
T_{regs}	Regulatory T cells

CHAPTER I

INTRODUCTION

1.1 Introduction to Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is a prototypical systemic autoimmune disease and it affects multiple organs and organ systems throughout the course of the disease(1). According to the Lupus Foundation of America there are 1.5 million SLE patients in America and more than 5 million patients worldwide. SLE has been a well-known immune disorder for more than a hundred years and the first published report of lupus dates back to 1856. It was later in the nineteenth century that the neoclassical era of lupus research began when Kaposi first described the systemic nature of the disease in 1872. Till now there is no treatment for SLE.

SLE is characterized by a wide variety of clinical symptoms which may vary from one individual to another. Such a wide array of symptoms makes the early diagnosis of SLE and the management of its clinical manifestations very difficult. In order to help in the early and easy diagnosis of SLE, the American College of Rheumatology has put

1.	Malar rash: butterfly-shaped rash across cheeks and nose
2.	Discoid (skin) rash: raised red patches
3.	Photosensitivity: skin rash as result of unusual reaction to sunlight
4.	Mouth or nose ulcers: usually painless
5.	Arthritis (nonerosive) in two or more joints, along with tenderness, swelling, or effusion. With nonerosive arthritis, the bones around joints don't get destroyed.
6.	Cardio-pulmonary involvement: inflammation of the lining around the heart (pericarditis) and/or lungs (pleuritis)
7.	Neurologic disorder: seizures and/or psychosis
8.	Renal (kidney) disorder: excessive protein in the urine, or cellular casts in the urine
9.	Hematologic (blood) disorder: hemolytic anemia, low white blood cell count, or low platelet count
10.	Immunologic disorder: antibodies to double stranded DNA, antibodies to Sm, or antibodies to cardiolipin
11.	Antinuclear antibodies (ANA): a positive test in the absence of drugs known to induce it.

forth a list of eleven characteristic symptoms of SLE **(Table 1.1)** and an individual is considered to have active disease if he or she is positive for any four of the eleven symptoms. Some of the most common symptoms are- Malar or the Butterfly rash, serum antibodies against nuclear antigens (dsDNA, histone and chromatin), kidney disorder and arthritis (1). The American College of Rheumatology has also developed a system for measuring the severity of the disease known as SLE.

Table 1.1 Clinical symptoms of SLE.

http://www.lupusresearchinstitute.org/lupus-facts/lupus-diagnosis

Disease Activity Index (SLEDAI) which is based on the classical symptoms used for the diagnosis of SLE. There are certain other disease indexes which are in clinical use to

predict long term mortality from lupus like disease. A significant gap in the knowledge of pathogenesis of SLE is the lack of early disease predicting markers.

1.1.2 Clinical manifestations of SLE

SLE earns the title of a prototypic systemic autoimmune disease from the fact that the disease can affect a wide array of organ systems. As shown in **Table 1.1**, SLE can manifest in many different forms. SLE is a chronic immune disorder characterized by systemic inflammation leading to renal, dermatological, hematological, cardiovascular, neuropsychiatric and musculoskeletal disorders **(Figure 1.1)**. The distribution of the symptoms among the affected population is variable and the extent of organ damage also varies from patient to patient. SLE proves to be a very complicated disease to treat as it follows a remitting and relapsing course. Depending on the organ/organ system involved in the pathology of SLE, it can be fatal in many cases.

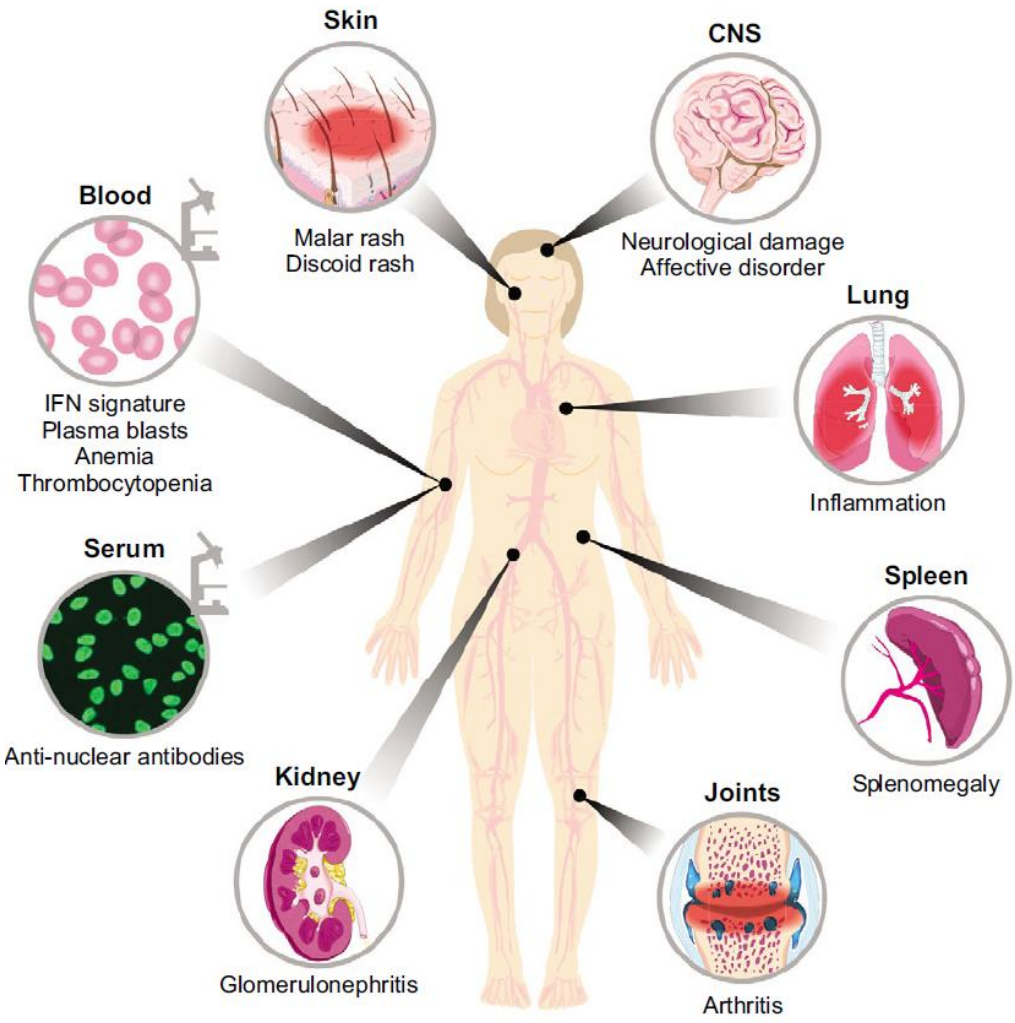

Figure 1.1 Clinical manifestations of SLE: Various organs and organ systems are affected in SLE leading to different symptoms and organ damage. *Adapted from (2).*

One of the prime examples of fatal organ pathologies is the renal failure in SLE patients due to deposition of IgG immune complexes (IC) within the glomeruli followed by complement fixation. Among lupus patients SLE is the most common cause of death, occurring in 34%, followed by infection (22%), cardiovascular disease (16%), cerebrovascular disease (6%), and cancer (6%) (3). SLE has been shown to be the

number one cause of heart failure in young women. To summarize, SLE is a multi-faceted disease with various manifestations and organ involvement and can often lead to fatality.

1.2 Current therapies for SLE and Limitations:

SLE is a very heterogeneous autoimmune disease and presents a challenge to be clinically treated. Advances in understanding the molecular, genetic, and pathological aspects of SLE in animal models have improved the treatment regimens in recent times but there are still many complexities in treating the human disease. Therapies for treating SLE can be broadly divided into two major categories - **1)** *Therapies aimed at systemic immune-suppression* and **2)** *Therapies targeting specific cells and molecules of the immune system* (4).

1.2 (A) Systemic Immunosuppression:

Common treatment regimens for SLE involve the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as– naproxen, ibuprofen or, corticosteroids like prednisone targeted at symptomatic relief during mild flairs of the disease. There have been studies which show a beneficial effect of anti-malarial drugs used in combination with NSAIDs in preventing and ameliorating mild manifestations of SLE (5,6). Other more aggressive therapies for severe SLE manifestations involve stronger and broader spectrum immune-suppressive agents like cyclophosphamide (7), azathioprine, Micophenolate mofetil (MMF) and methotrexate (8). Most of these drugs target pathways of de novo synthesis essential for cell division and thereby minimize the generation of new auto-reactive T and B cells. Some of these drugs are even used in a combinational manner to improve

outcomes in patients. For example, MMF and cyclophosphamide used in combination has been shown to improve renal function in SLE patients (9). However, there are side effects of these therapies which predominantly manifest in form of leukopenia (decrease in numbers of circulating leukocytes), gastro-intestinal disorders, oral ulcers, nausea, vomiting and susceptibility to opportunistic infections (4). Different therapies have shown various levels of success but there is a lack of a set treatment regime with wide spread benefits and fewer side effects.

1.2 (B) B cell targeted therapies:

In recent years, the use of monoclonal antibodies against specific immune components, cytokines and cell types has emerged as alternative therapies for SLE. The advantage of these therapies over systemic immune-suppression is a higher degree of specificity and fewer side effects. The most attractive target for cell based therapies over the years has been B cells as B cells produce pathogenic auto-antibodies leading to most of the complications seen in SLE patients. Most of the B cell targeted therapies are either aimed at B cell surface specific antigens for directly eliminating pathogenic B cells or modulating B cell activation and survival signals. One of the earliest attempts at B cell therapies came in 1997 when rituximab, a chimeric murine/human monoclonal antibody (mAb) against B cell surface specific antigen CD20 was approved by the US Food and Drug Administration (FDA) for use in humans (10,11). In the following years other anti-CD20 mAb like atumumab, ocrelizumab and veltuzumab came into existence. Unfortunately, anti-CD20 mAb either have failed to clear the safety standards to be used in humans or failed to meet the end point requirements (4).

Another approach to B cell based therapies are mAb targeting the B cell receptor (BCR) or co-stimulatory molecules. Eprauzumab is a mAb against B cell surface specific antigen CD22 which modulates BCR signaling causing a decrease in B cell numbers by 35-45% (12) and has been shown to have positive results in phase IIb SLE trial. Abetimus is a mAb which binds to BCR and induces anergy (functional inactivation) of B cells. However, none of these mAb has not been approved for clinical use due to lack of sufficient data or efficacy (4,13).

1.2 (C) B cell survival signal targeted therapies:

The last category of B cell directed mAb are aimed at blocking B cell survival signals and co-stimulatory molecules required for activation and differentiation of B cells into antibody producing plasma cells. B cell activating factor (BAFF) is a soluble B cell survival factor that binds to its receptors TACI (transmembrane activator and calcium-modulator and cyclophilin-ligand interactor), B-cell maturation antigen and BAFF receptor. Increased BAFF levels have been associated with disease activity in SLE patients and have been shown to be correlated to disease severity (14-16). Belimumab is a humanized mAb against soluble BAFF that prevents the binding of BAFF to its receptors on B cell surface. Belimumab showed very promising results in clinical trials and was very well tolerated with a high efficacy. There was a marked decrease in the total number of B cells, plasmablasts, and IgG, IgA, IgM and IgG anti-ds DNA titers after treatment with Belimumab (17,18). Belimumab earned the distinction of being the first FDA approved (2011) therapy for SLE in over 50 years. Another mAb limiting B cell survival is Atacicept, directed against TACI, the receptor for BAFF and APRIL

(another B cell survival signal) leading to reduced serum Ig; however, one side effect is increased susceptibility to infections in treated individuals (19).

1.2 (D) Co-stimulatory molecule targeted therapies:

Naïve B cells need co-stimulatory signals from T cells to differentiate and proliferate into plasma cells, making co-stimulatory molecules a viable target for SLE therapy. B cells need engagement of CD40 cell surface antigen with its ligand CD40 ligand (CD40L) in order to undergo a germinal center reaction and become antibody secreting plasma cells. Blocking of the CD40-CD40L pathway was shown to be beneficial in murine models of lupus (20,21). Additionally, mAb against CD40L have been used in clinical trials of SLE (19,22) but have not been sufficiently effective. Further studies and trials are required to understand the exact mechanism of this therapy. Alternatively, blocking of another co-stimulatory pathway involving B-cell coligands B7-1 and B7-2 with a fusion protein of extracellular domain of cytotoxic T lymphocyte-associated antigen 4 receptor (CTLA4) has shown promising results in mouse models of lupus (23). Figure.2 summarizes the different B cell directed approaches with their targets.

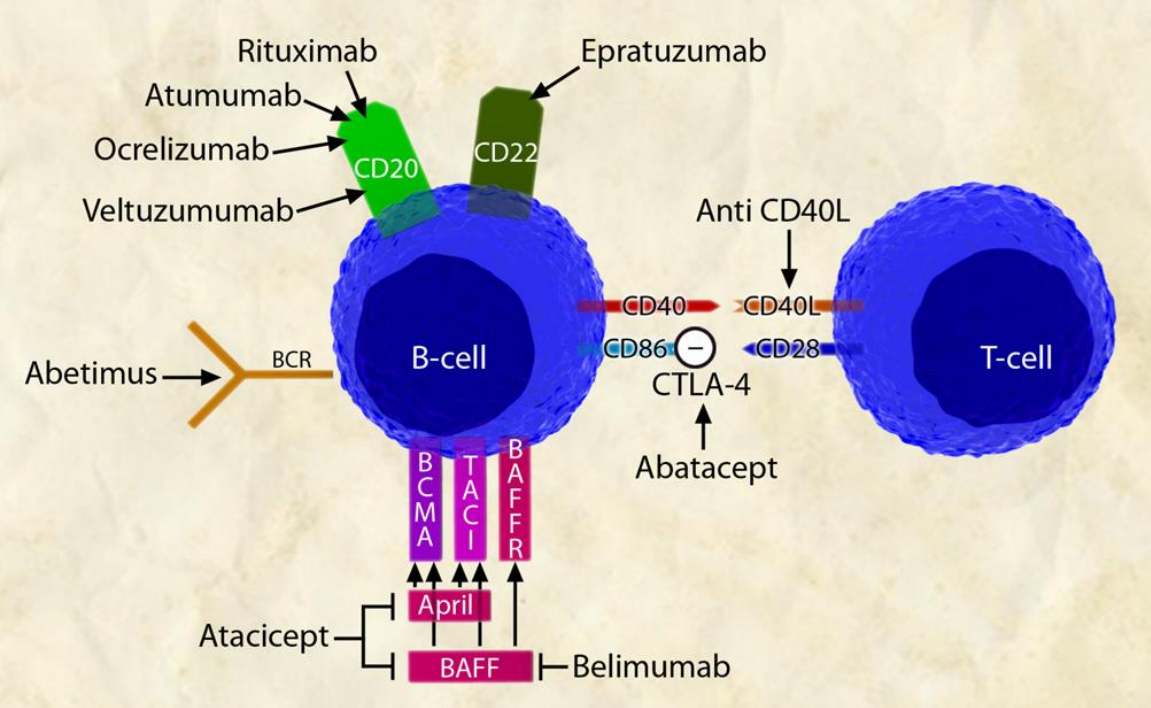

Figure 1.2 B cell based therapeutic targets for treatment of SLE. B cell costimulatory molecules and receptors for the survival factors BAFF and APRIL are shown as well as cell surface molecules being targeted therapeutically. The therapeutic agents are identified along with their targets. *Adapted from (4).*

1.2 (E) Alternative therapies:

There are studies showing the efficacy of anti-cytokine therapies in treatment of lupus patients. Neutralizing disease promoting cytokines TNF, IL-1, IL-10, IFN-α and IL-6 has shown promising results in mouse models of lupus and other autoimmune diseases (reviewed in detail in (4)). Additionally, there are studies showing the beneficial effect of blocking specific kinases involved in immune cell function as a target for treating autoimmune diseases. Specifically, use of small molecule inhibitors of Spleen tyrosine kinase (Syk), responsible for aberrant T cell signaling in lupus, has been shown to suppress disease in lupus prone (NZBxNZW) F1 mice (24). There are ongoing clinical trials for Syk inhibition and it could be a promising therapy for SLE (25).

To summarize, there are various emerging therapeutic targets for treatment of SLE, but only a few have shown promise with high efficacy and few side effects. The most common side effect of current therapies is an immune-compromised state of the treated individuals leaving them highly susceptible to opportunistic infections and poor quality of life. There is no permanent cure for SLE to date and there is a need to develop more specific therapies with lesser side effects.

1.3 Overview of autoimmunity and triggering mechanisms:

Classically, autoimmune diseases are defined as the response to non-foreign antigens mediated by T cells and antibody secretion by B cells. Autoimmunity could manifest in an organ or tissue specific manner or can affect multiple organs and organ systems. According to National Institute of Allergy and Infectious Disease (NIAID), there are 80 defined autoimmune diseases and the underlying cause of all the autoimmune disease is a

dysregulated immune system. Autoimmunity arises from the dysregulation of the immune system where the body's defense mechanism goes into uncontrolled overdrive and attacks self-tissue. It is notable that both the innate (non-specific) and acquired (specific) branches of the immune system contribute to the development of autoimmunity with specific cell types playing distinct roles *(discussed in more detail in section 1.4).* Hence, it becomes critically important for the immune system to be able to precisely differentiate between self and non-self-antigens. The importance of identifying self and non-self was recognized as early as the 1950s when Medawar and colleagues demonstrated with elegant experimental approaches the very first evidence of *immune tolerance* (26). Any breach in immune tolerance can lead to immune-dysregulation and autoimmunity.

Several theories and factors explain the initiation of autoimmunity resulting from immune dysregulation. In this section, different triggers of autoimmunity and the underlying causes will be discussed.

1.3.1 Tolerance to self-antigens:

One of the most fascinating features of the immune system is it's ability to clearly distinguish between self-and non-self-tissue, which provides critical cues to immune cells as when to mount an immune response and when not to. It is appreciated that even after maintaining a tremendous ability to mount an immune response against an immense number of foreign antigens; the immune system preserves its specificity and tolerance towards self-antigens. The most important cell types are equipped with antigen-specific receptors and have the ability to design their receptors for a wide array of antigens,

notably T cells and B cells. Most of the time the generation, remodeling and retention of the T cell receptor (TCR) and BCR and subsequent deletion of self-reactive T cells and B cells works flawlessly, but there are occasions when the system is leaky and may lead to non-desirable outcomes in terms of self-reactive T and B cells. T cells and B cells, on recognizing an antigen and receiving the appropriate co-stimulatory signals, can undergo clonal expansion and mount an immune response and recruit innate immune cells to take part in the ongoing immune response. If the T and B cells encounter and recognize a self-antigen, it can lead to deleterious anti-self-immune response that can prove to be very damaging. Thus it becomes critically important to have safety check points at the developmental level for elimination of self-reactive T and B cells.

1.3.1 (A) Central tolerance

The process of central tolerance is aimed at eliminating any self-reactive lymphocytes (T and B cells) at an early developmental stage. Although both T cells and B cells arise from a common Hematopoietic Stem Cell (HSC), their development takes place in different lymphoid tissues (27,28). T cell development takes place predominantly in thymus and B cells undergo development in the bone marrow (BM). Thus, the process of central tolerance takes place in lymphoid tissues – thymus (T cells) and bone marrow (B cells).

During T cell development in the thymus, T cells undergo a sequential series of differentiation steps. T cells start as $CD4^-$ $CD8^-$ (Double negative) which become $CD4^+$ $CD8^+$ (Double positive) and finally mature into $CD4^+$ or $CD8^+$ (Single positive) cells (29). There are distinct selection processes that the developing T cells must undergo. Firstly, the double positive ($CD4^+$ $CD8^+$) T cells undergo positive selection. Only the T

cells that can recognize either peptide/MHC class I or peptide/MHC class II complexes in the thymic cortex receive a positive signal to continue in the maturation process, while the remaining T cells with a non-viable TCR are eliminated by the process of apoptosis (28). After undergoing positive selection for a functional TCR, T cells are either committed to CD4 or CD8 lineage and later on form the majority of peripheral T cell population (30). The next step in T cell development, negative selection, is very critical to the induction of tolerance and involves elimination of T cells bearing a self-reactive TCR. The most important molecule in negative selection of T cells is the transcription factor autoimmune regulator (AIRE). AIRE is expressed by medullary thymic epithelial cells and it controls the expression of numerous self-antigens in medullary thymic epithelial cells (31,32). These self-antigens are presented to developing T cells in complex with MHC and T cells with high reactivity to self-antigen/MHC complex are deleted (33,34). Some T cells with intermediate reactivity to self-antigen/MHC complexes evade the negative selection process and go on to become regulatory T cells (T_{regs}) (described later in section 1.5.2) (32,33). Hence, AIRE plays a very important role in negative selection of self-reactive T cells, development of T_{regs} (regulatory T cells) from some self-reactive T cells and induction of central tolerance. The critical role of AIRE in maintenance of central T cell tolerance has been shown in both humans and mouse models where a mutation or deficiency of AIRE leads to severe autoimmune diseases (35).

B cell central tolerance occurs in BM during B cell development and differentiation. The central B cell tolerance is mediated either by the deletion of self-reactive B cells or by receptor editing (36,37). Immature B cells reacting with high affinity to self-antigen

undergo apoptosis (deletion). This deletion mechanism may be crucial when all receptor editing options are exhausted (37). B cells express recombination activating genes (Rag) which are responsible for generation of BCR diversity and receptor editing. After generation of a viable BCR, immature B cells in the bone marrow cease to express Rag genes. Immature B cells that encounter multivalent self-antigens revert to pre-B cell-like phenotype, re-express Rag genes, and induce κ light chain gene rearrangements (if necessary, λ). New Vκ-Jκ rearrangements, involving upstream V segment and downstream J segment, delete the original Vκ-Jκ rearrangement that had produced a self-reactive light chain, resulting in newly generated B cells that have a novel light chain that is no longer reactive to self-antigen. These immature B cells then migrate to the periphery where they mature into IgM- and IgD-bearing B cells (38-40). The importance of Rag genes in B cell central tolerance is confirmed by studies showing that mutations in Rag genes correlate with development of autoimmunity in humans as well as mouse models (37).

Hence, the process of central tolerance ensures that the auto-reactive T and B cells are eliminated during development; but still, some of the auto-reactive lymphocytes escape negative selection and enter the periphery. There are further check points to eliminate such cells in the periphery but sometimes the breach of central tolerance can lead to autoimmunity.

1.3.1 (B) Peripheral tolerance:

There are certain immune mechanisms that are in place to deal with the auto-reactive B and T cells that evade central tolerance check points and enter the periphery. The main

players in T cell peripheral tolerance are T_{regs}. T_{regs} are characterized by the expression of transcription factor Foxp3 which mediates their immune-suppressive function. T_{regs} deprive effector T cells of antigens by forming long-lasting interactions with antigen presenting dendritic cells (41) and thereby dampen the immune response. Mutations in Foxp3 gene have been associated with development of autoimmunity in both humans and mice (35,42).

B cell peripheral tolerance is mediated by induction of anergy, clonal deletion of auto-reactive mature B cells, direct suppression by T_{regs} and clonal ignorance (37). However, anergy seems to be the most dominant mechanism involved in B cell peripheral tolerance. Anergy is the process of maintaining auto-reactive B cells in a state of hyporesponsiveness and making them incapable of reacting to self-antigens. B cell anergy is mostly mediated by the inability of self-reactive B cells to respond to antigen coupled to BCR. The most common mechanism for B cell anergy is the lack of co-stimulation by T cells(43). Defects in silencing auto-reactive B cells in the periphery may lead to adverse outcomes and autoimmunity.

1.3.2 Mechanisms involving innate immune cells:

Innate immune cells form a very important part of the immune system and play an instrumental role in generating an appropriate immune response. Innate immune cells perform various functions like immune surveillance, antigen presentation and clearance of cellular debris. Dysregulation of innate immunity can lead to aberrant activation of acquired immune cell subsets and may lead to autoimmunity (44). In this section the role

of innate immune cells and the underlying mechanisms that may trigger autoimmunity is discussed.

1.3.2 (A) Dendritic cells and toll like receptors (TLRs):

Dendritic cells (DCs) are the most potent antigen presenting cells of the immune system and play a crucial role in mounting immune responses. One of the striking features of DCs is the expression of membrane bound Toll-like receptors (TLRs) which are pattern recognition receptors (PRRs) capable of responding to both endogenous as well as exogenous antigens. Currently 13 members of TLR family have been identified, out of which TLR1 to TLR10 are functional in humans (45). TLRs are capable of recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (46). PAMPs are evolutionarily conserved exogenous molecules like lipopolysaccharide (LPS), peptidoglycan and bacterial and viral nucleic acids. DAMPs on the other hand are endogenous molecules like heat shock proteins which are released during tissue injury and cell death (47). TLR3, TLR7, TLR8 and TLR9 are found on the endosome in DCs (dendritic cells) and recognize viral nucleic acids. Upon binding to their respective ligands TLRs initiate signaling cascades resulting in DC activation, upregulation of co-stimulatory molecules (CD80, CD86 and MHCII) and release of pro-inflammatory cytokines (IFN-α, IL-6) capable of inducing T cell activation (48). It has been shown in a number of autoimmune diseases, like SLE, type I diabetes and multiple sclerosis, that over-stimulation of TLRs can lead to abnormal activation of T and B cells, resulting in autoimmunity (44). Specifically, SLE is characterized by production of anti-nuclear antibodies and the presence of circulating immune complexes containing DNA

and RNA. It has been shown that CD32-mediated uptake of these immune complexes containing DNA and RNA by DCs and the subsequent activation of TLR7 and TLR9 play an important role in pathogenesis of SLE (49,50).

Additionally, DAMPs can also stimulate TLRs and there is a strong possibility that it can lead to autoimmunity via DC-mediated activation of T and B cells in response to mechanical tissue injury or necrosis (51).

1.3.2 (B) Defective clearance of cell debris:

As discussed above, circulating immune complexes and DAMPs can lead to over-stimulation of DCs, resulting in a break of tolerance and induction of autoimmunity. Hence, efficient clearance of cell debris is required to make sure that there is not an excess of TLR stimulants present in the system. There is defective clearance of apoptotic bodies in mouse models of SLE (52). Also tissue damage resulting in necrosis leads to abundance of auto-antigens in circulation. Macrophages are the major scavenging cells of the immune system responsible for clearing cellular debris and apoptotic bodies. It has been shown in SLE patients that there is defective clearance of apoptotic bodies by macrophages which in turn causes prolonged exposure to auto-antigens and predisposes one to auto-antibody production (53).

1.3.3 Environmental factors:

Autoimmunity could be triggered by environmental factors, including exposure to microorganisms and diet. Although the exact mechanisms are not well understood, it has been shown in various animal models that modulation of gut microbiota can alter the susceptibility to autoimmune diseases (54). The composition of gut microbiota can have pro-inflammatory or anti-inflammatory effects depending on the host immunological state (55). In a mouse model of experimental autoimmune encephalomyelitis (EAE) *Bacteroides fragilis* polysaccharide A protects from disease by inducing T_{regs} (56). Similarly, in (NZWxBXSB)F1 model of lupus, caloric restriction has a protective effect on lupus-like disease development (57). On the other hand, there is emerging evidence that engagement of TLRs with gut microbiota DNA can alter the course of autoimmunity (58). Another interesting theory linking the exposure to microorganisms and development of autoimmunity is the ***Hygiene Hypothesis***. This theory postulates that the increased incidence of allergic and autoimmune disorders in developed countries could be an outcome of a lack of exposure to microorganisms that co-evolved with the human population. A sudden exposure to such microorganisms may lead to induction of undesired immunological responses leading to autoimmunity (59). As diet and composition of gut microbiota go hand in hand it is tough to differentiate the role of each factor in modulating immunity.

Molecular mimicry has also been associated with induction of autoimmunity as it has been shown that a lot of viral antigens mimic SLE antigens and can lead to relapse or even initiation of SLE (60,61). Specifically, an Epstein - Barr virus (EBV) infection has been associated with increase in SLE incidence. It has been demonstrated that antibodies

against EBV antigens have cross reactivity to dsDNA which is a major SLE antigen. In murine models immunized by EBV antigens the production of anti-dsDNA antibodies was enhanced (62,63). These studies provide a clear link between molecular mimicry and autoimmunity but further studies are required to elucidate exact mechanism and correlation to human autoimmune diseases and infections.

Also, the role of genetics cannot be overlooked in shaping the immune system and susceptibility to autoimmunity (discussed in detail in section 1.6).

To summarize, autoimmunity is a very complex process and the exact underlying mechanisms triggering autoimmunity are not clear. An autoimmune disease could manifest as a result of any of the above mentioned triggers or can be an outcome of a combination of multiple factors.

1.4 Sex Bias in SLE:

The majority of the systemic autoimmune diseases have a strong female bias but the actual basis of the sex bias in autoimmunity is not well understood. In fact studies have shown that of all the autoimmune-disease affected individuals, more than 75% are females (64,65). SLE has a strong female bias, with a female to male ratio of 9:1 in human population. SLE is most prevalent in young women during the child bearing years (15-45years) and the incidence of the disease goes down in menopausal women (66). It is to be noted that in prepubescent years there is a female bias in SLE but the ratio of female to male patients is 3:1. Although fewer men develop SLE, male patients present with a more severe form of the disease involving a higher incidence of seizures, more severe

renal disease as well as cardiorespiratory disorders as compared to female patients (66,67). Over all this suggests that the development and pathogenesis of SLE is different between males and females.

1.4.1 Mouse models to study SLE:

Many murine models of SLE exist and some of them show a similar sex bias in disease development as observed in humans. Commonly used mouse models of lupus are - (NZB x NZW)F1 hybrid, B6.Nba2, B6.Sle123, MRL/*lpr*, and BSXB/*Yaa* (68) . All these mouse strains spontaneously develop a lupus like disease and mimic a variety of symptoms present in human SLE. One of the widely studied spontaneous murine models of lupus, the (NZBxNZW) F1 mouse has a strong female bias to the development of lupus-like disease. These mice are genetically susceptible to develop a lupus like disease where 100% of the females develop disease by 12 months of age whereas only 30% of male mice develop disease (69). Interestingly, in the (NZBxNZW) F1 mouse model, lupus like disease is characterized by elevated levels of circulating serum autoantibodies to nuclear antigens chromatin, histone and dsDNA. These mice also develop renal disease marked by IgG immune complex (Ig-IC) deposition within the kidney glomeruli. Glomerulonephritis and the extent of renal damage can be measured in terms of proteinuria (70,71). (NZBxNZW)F1 is a spontaneous and multigenetic model of SLE and closely resembles the sex bias observed in humans. Additionally, (NZBxNZW) F1 mice mimic most of the important symptoms of human disease which makes it a valuable tool to study lupus. All the research described in this thesis has been performed using the (NZBxNZW) F1 mouse model of lupus.

1.4.2 Role of sex hormones in gender bias in SLE:

The fact that 9 out of 10 SLE patients are female in their child bearing years and that the incidence of disease is lower in prepubescent and menopausal women strongly suggests the role of female sex hormones in the pathophysiology of SLE. The incidence of SLE is the highest in women during the time of their life when they are exposed to maximum levels of female sex hormones. Also the noticeable lower prevalence of SLE in male populations indicate that there is a protective role of androgens in the development of SLE.

1.4.2(A) Pathological role of Estrogen in SLE:

Early studies performed on (NZBxNZW) F1 mice showed that ovariectomy of female (NZBxNZW) F1 mice protected them from developing a lupus like disease and treatment of castrated male mice with estrogen leads to severe lupus-like disease (72,73). B cells are central in SLE as they produce auto antibodies leading to disease pathology. Several studies have documented the role of estrogen on B cell development, differentiation and tolerance. It has been shown in a estrogen driven model of lupus that elevated levels of estrogen lower the threshold for negative selection by up regulating Bcl-2 (anti-apoptotic molecule) during B cell development, breaching central tolerance of B cells, thereby increasing the number of auto reactive B cells in the periphery (74,75). Additionally estrogen can enhance the maturation of B cells (76) and selectively impair the negative selection of high-affinity DNA reactive B cells leading to higher numbers of mature DNA reactive B cells (77). Estrogen has also been shown to increase the expression of the co-stimulatory molecule CD40L on T cells and B cells from lupus patients (78,79). Hence, estrogen not only directly pushes B cells towards a pathogenic phenotype, but

also aids B cell differentiation and maturation *via* up regulating co-stimulatory signal from T cells.

1.4.2(B) Protective role of testosterone in SLE:

Testosterone has a broad immune-suppressive effect on the immune system as is evident from the fact that males are more susceptible to infections and cancers as compared to females (80). Specifically, in SLE the protective role of testosterone was clearly demonstrated in 1970s by Talal and colleagues. In sex hormone manipulation studies in lupus prone (NZBxNZW) F1 mice it was shown that male mice were protected from disease development by the presence of testosterone as castration resulted in the development of lupus-like disease symptoms similar to those observed in age-matched female (NZBxNZW) F1 mice. Even more interesting, female (NZBxNZW) F1 mice with severe disease showed reduced disease severity and prolonged survival upon treatment with testosterone (72,73,81). The protective role of testosterone is not only limited to SLE but it has been shown to play a protective role in other autoimmune diseases like-EAE and rheumatoid arthritis (RA) (82,83). Likewise, testosterone-replacement therapy of Klinefelter's Syndrome patients led to decreased serum antibody and cytokine levels and decreased T and B cell levels (84). It has been documented that SLE patients have lower circulating levels of testosterone and higher aromatase activity which can convert testosterone to estrogen (85).

It is evident that male and female sex hormones play a significant role in sexual dimorphism observed in SLE and other autoimmune diseases. Estrogen drives autoimmunity while testosterone is protective. To a large extent the disease driving

mechanisms and effects of estrogen on immune cells are well understood. But there is gap in knowledge for many years as to how testosterone exerts a protective effect in SLE. The mechanisms of testosterone driven protection from SLE are not well understood and have been underexplored.

1.5 Key cellular components in lupus:

1.5.1 Role of B cells:

Most of the organ damage seen in lupus is mediated by auto-antibodies produced by B cells, so B cells can be called the drivers of lupus pathogenesis (86). B cell hyperactivity is a hallmark of SLE. Naïve B cells from mouse models of SLE have been shown to respond very strongly to BCR stimulation as compared to naïve B cells from non-autoimmune mouse strains (87). B cells from lupus patients have a high expression of CD40L, generally expressed only by T cells, indicating that lupus B cells can activate other naïve B cells (88). Moreover, the emerging role of B cells as cytokine producing and antigen presenting cells can also contribute to their role in lupus progression (89). Additionally, there is an accumulation of auto-reactive B cells in the marginal zones of spleens in mouse models of SLE (89,90). These auto-reactive B cells have a low threshold for tolerance and can play a prominent role in initiating autoimmunity (90). A regulatory B cell subset producing IL-10 (B_{regs}) has been identified but the role of this B cell subset is not well understood in the development of SLE (91,92).

1.5.2 Role of pDCs:

Plasmacytoid DCs (pDCs) were first identified as $CD11c^-$ immature DCs in human peripheral blood (93). As compared with conventional DCs, pDCs express lower levels of MHCII, lower levels of co-stimulatory molecules and a reduced ability to stimulate T cells (93). In contrast, pDCs are the dominant interferon-producing cells in response to most viral infections. In fact pDCs have been shown to produce 200-1000 times more IFN-α, in response to microbial challenge, compared to any other cell type, conferring these cells an important role in anti-viral and anti-tumor immune responses (94,95). There have been several studies showing the crucial role of IFN-α in development of lupus like disease in mouse models. In fact IFN-α has been associated with development of auto-antibodies and IFN-α receptor deficiency has been shown to protect lupus prone mice from developing disease (96,97). There is an elevated level of circulating IFN-α in human SLE patients which correlates with disease severity and anti-dsDNA antibodies (98,99). Also, peripheral blood cells from SLE patients present with a typical gene expression pattern known as an "Interferon Signature" where IFN-α inducible genes are upregulated (100,101). A recent study showed that depletion of IFN-α producing pDCs in lupus prone B6.Nba2 mice leads to reduced levels of anti-nuclear auto-antibodies (102). Since, pDCs are dominant producers of IFN-α, we can speculate that they may play a pivotal role in SLE pathogenesis.

1.5.3 Role of neutrophils:

Neutrophils are innate immune cells of myeloid origin and arise from the common myeloid progenitor (CMP) in the BM in response to specific stimuli including both cytokines and growth factors (103). Neutrophils are the most abundant circulating

immune cells and form the first line of defense against microbial and fungal infections. These cells are short-lived and contain granules rich in anti-microbial proteins to kill invading pathogens (104). The disease driving role of neutrophils in SLE has been extensively studied and well documented (105), but in recent years there have been studies exploring the anti-inflammatory properties of neutrophils in SLE and a variety of other autoimmune disorders (106). In the following section, the pro and anti-inflammatory role of neutrophils will be discussed in detail.

1.5.3 (A) Pro-inflammatory role of neutrophils:

The role of neutrophils in driving SLE has become more evident in recent years and various neutrophil driven phenomena have come to light that can aid SLE progression. Neutrophils are potent producers of cytokines that can modulate both T cell and B cell function and differentiation. Neutrophils produce IL-6, BAFF and APRIL, powerful B cell regulators associated with B cell development and antibody production (106). IL-6 manipulation has been shown to affect lupus pathogenesis in mouse models of SLE (107-109). BAFF is a B cell survival signal and it has been reported that neutrophil derived BAFF leads to elevated T and B cell responses in lupus prone mice (110). APRIL is a plasma cell survival signal and BM neutrophils from lupus patients produce APRIL (111). Thus, neutrophil derived cytokines can aid B cell differentiation and development at an early stage and prolong survival of antibody producing plasma cells. Another important cytokine in SLE pathogenesis is IL-21, which is required for GC formation and antibody production (112). Neutrophils can produce IL-21 under chronic inflammatory conditions and lead to T cell independent B cell activation and differentiation in SLE

(113). Finally, neutrophils can also produce IL-17A which has been implicated in development of SLE and other autoimmune disease (114).

Another interesting area of emerging research regarding the role of neutrophils in pathogenesis of SLE is NETosis. NETosis is a specialized process of neutrophil death where neutrophils form NETs (neutrophil extracellular traps) to trap pathogens and subsequently undergo death (115,116). NETs are loaded with anti-microbial proteins like myeloperoxidase (MPO) and LL37, along with histones and free DNA and are shown to induce IFN-α (key cytokine in SLE discussed in section 1.6) production *via* TLR9 crosslinking in pDCs (117). Recently, in SLE patients a subset of neutrophils termed as low density granulocytes (LDGs) have been shown to undergo spontaneous NETosis *in vitro* and produce IFN-α (118).

1.5.3 (B) Anti-inflammatory/immunosuppressive role of neutrophils:

The very first evidence for the existence of the anti-inflammatory neutrophils came from cancer studies. Ly6-G^+ and Ly6-C^+ immature myeloid cells were shown to infiltrate tumors and to suppress anti-tumor T cell responses in cancer patients (119). Since then there have been numerous studies defining the immunosuppressive function and mechanisms of these neutrophil like cells. Such cells have been named myeloid derived suppressor cells or MDSCs. The regulatory functions of MDSCs and the identification of MDSCs as potent immunosuppressive cells were first established in cancer models, where the cells act to suppress T cell mediated anti-tumor responses (reviewed in detail (120)). MDSCs are further divided into two different categories based on cell surface marker expression- Gr-1^{high}Ly-$6G^+CD11b^+$(granulocytic MDSCs) or Gr-1^{low}Ly-

6C^{+}CD11b^{+}(monocytic MDSCs) (121) **(Figure 1.3)**. MDSC subsets isolated from tumors have been shown to use reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), and TGF-beta as their main mechanisms of T cell suppression (121-124). The role of MDSCs in autoimmune diseases is currently emerging and it has already been shown in EAE and type I diabetes that MDSCs can suppress autoimmunity and alleviate disease symptoms (125,126).

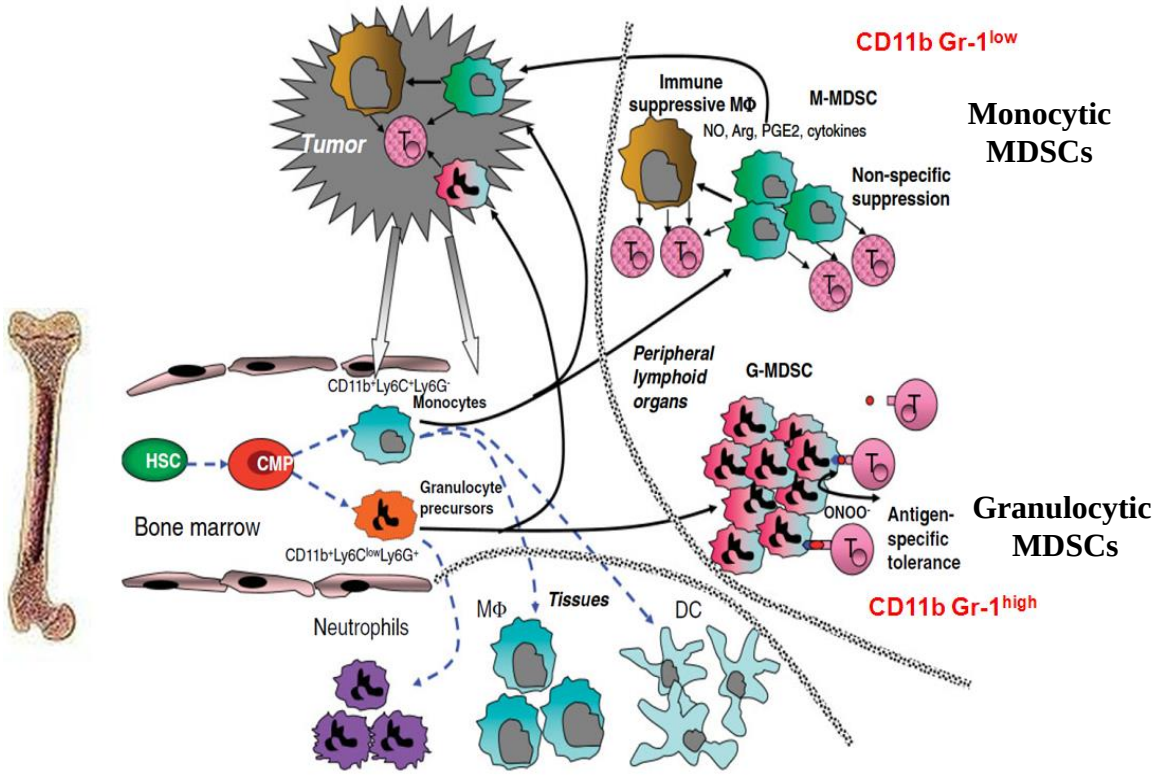

Figure 1.3 The pathways of MDCS development and differentiation in cancer. MDSCs develop from the CMP (common myeloid progenitor) in BM. MDSCs are classified as Monocytic MDSCs (Gr1^{low} CD11b^{+}) and Granulocytic (Gr1 ^{high} CD11b^{+}). *Adapted from (120).*

These studies encouraged us to explore the role of MDSCs like cells in lupus prone (NZB x NZW) F1 mice. Interestingly, we found elevated levels of Gr-1^{high}Ly- $6G^{+}CD11b^{+}$ and Gr-1^{low}Ly-$6C^{+}CD11b^{+}$ MDSC like cells in lupus prone male (NZB x NZW) F1 mice as compared to female (NZB x NZW) F1 mice and hypothesized that – **Higher of levels of Gr-1^{high}Ly- $6G^{+}CD11b^{+}$ and Gr-1^{low}Ly-$6C^{+}CD11b^{+}$ MDSC like cells in lupus prone male (NZB x NZW) F1mice may protect them from development of lupus like disease.** The results of the studies performed to test this hypothesis are discussed in detail in ChapterII **(Gr-$1^{high}CD11b^{+}$ Cells Suppress B cell Differentiation and Lupus-like Disease in Lupus-Prone Male Mice; Published in *Arthritis and Rheumatism*, 2013).**

1.5.4 Role of T cells:

T cells are indispensable for driving lupus like disease as T cells help is essential for formation of germinal centers and B cell differentiation into antibody secreting plasma cells (127,128). There are different functions associated with the various T cell subsets in the pathogenesis of SLE. This section will discuss the role of each T cell population in SLE.

1.5.4 (A) Role of T_H17 cells:

T_H17 cells are characterized by the expression of the transcription factor ROR-γt and the production of the pro-inflammatory cytokines IL-17, IL-21, IL-22, and IL-6 (129,130). T_H17 cells play an important role in protection against bacterial and fungal infections and clearance of pathogens (131,132). T_H17 cells have also been associated with the induction and development of autoimmune diseases, including SLE (114,132-134). Several studies have shown an increase in T_H17 cells in both murine models of lupus as

well as in patients with SLE. Additionally, the increased circulation of $CD4^+$ T_H17 cells correlates with disease activity in patients with SLE (131,135). The main cytokine produced by T_H17 cells, IL-17, works in accordance with BAFF, promoting B cell proliferation and differentiation into antibody secreting cells, and ultimately the production of autoantibodies (136). The T_H17 polarizing cytokine IL-6 is also increased in patients with SLE as opposed to healthy controls, and studies have shown that IL-6 plays a role in B-cell hyperactivity and autoantibody production (131,135,137). A dichotomy exists between levels of T_H1 and T_H17 in patients of SLE. The T_H1 cytokine, IFN-γ is shown to suppress T_H17 differentiation, and a deregulation between T_H17:T_H1 balance has been observed in patients with SLE (131,135). In support of this, a significant down-regulation of IFN-γ and reduced levels of T_H1 cells have been reported in patients with SLE (131,135,138). However, increased levels of IFN-γ were observed in patients with active SLE (135). Overall, T_H17 cells play a disease driving role in SLE by mostly promoting auto-antibody producing B cells.

1.5.4 (B) Role of T_H1 cells:

T_H1 cells play a crucial role in protecting an individual from infections by bacteria, fungi, and viruses (132,139-141). The pro-inflammatory cytokines produced by these cells, IFNγ and TNFα/β, stimulate innate and cell-mediated responses, resulting in the clearance of pathogens (142). T_H1 cells however have been associated with autoimmune diseases such as type I diabetes and RA (143,144). Studies have shown that both levels of T_H1 cells as well as levels of IFN-γ are decreased in patients with SLE, leading to an increase in T_H17 cells and a propensity towards disease (131,135,138). Other studies have

shown that, among patients with lupus nephritis however, a predominance of T_H1 cells is observed and promotes renal inflammation (145). Thus, the role of T_H1 cells in the progression of lupus is not clear.

1.5.4 (C) Role of T_H2 cells:

The function of T_H2 cells is associated with activating humoral immunity and promoting IgG1 and IgE class switching, protecting against extracellular pathogens such as helminthes (132,146). T_H2 cells secrete IL-4, IL-5, IL-6 as well as the pleiotropic cytokine IL-10. Interleukin-10 is best known for its anti-inflammatory effects and enhancing the survival and differentiation of B cells (147,148). Patients with SLE have been shown to have increased plasma levels of IL-10 (135,137). IL-10 has been shown to be over produced by B cells and monocytes in patients with SLE (149), and may act in an autocrine or paracrine fashion resulting in B cell hyperactivity. Levels of plasma IL-10 were also shown to correlate with overall disease activity (135). The effects of IL-10 on lupus pathogenesis may result from increased TLR-9 expression on B cells, which has been shown to correlate with disease activity and anti-nuclear autoantibodies in SLE patients (150).

1.5.4 (D) Role of T_{regs}:

T-regulatory cell (T-regs) are characterized by the expression of the transcription factor FoxP3 and surface markers $CD4^+CD25^+$. T-regs are known to produce the cytokines TGF-β and IL-10 (132). It has been speculated that T-regs suppress autoreactive effector T cells and that a decrease in levels of T-regs may result in the development of autoimmune disease; however the role of T-regs in lupus is not fully understood (138).

Studies conducted with SLE patients showed a decrease in TGF-β levels both in plasma and within the periphery (135) . The increased presence of IL-6 within these patients may be inhibiting the induction of T-regs and instead promoting T_H17 differentiation and lupus initiation (135).

1.5.4 (E) Role of T_{FH}:

T follicular helper cells (T_{FH}) are a pro-inflammatory subset of $CD4^+$ T cells that are required for germinal center (GC) formation and plasma cell differentiation. Germinal centers are the sites within secondary lymphoid tissue (spleen and lymph node) where mature B cells interact with T cells and undergo proliferation and differentiation, affinity maturation and class switching to generate a T cell dependent antibody response. The formation of GCs **(Figure 1.4)** is central to mounting an antibody mediated immune response and generation of long lived plasma cells (reviewed in detail in (151). T_{FH} cells are defined by the production of IL-21 which promotes GC formation and B cell differentiation, expression of the chemokine receptor CXCR5 which allows them to relocate to B cell follicles, expression of the transcription factor Bcl-6 and the costimulatory receptor ICOS, and high expression of the inhibitory receptor PD-1 (152). Within the GC, T_{FH} cells promote somatic hypermutation and selection of high-affinity B cells followed by differentiation of long-lived memory or plasma cells. This process ensured the development of long-lived humoral immunity (112). T_{FH} cells have been shown to be dysregulated in SLE patients (153,154). Increased levels of T_{FH} cells caused by T_{FH} dysfunction results in increased GC formation and differentiation of B cells into antibody producing cells, leading to the production of autoantibodies (154,155).

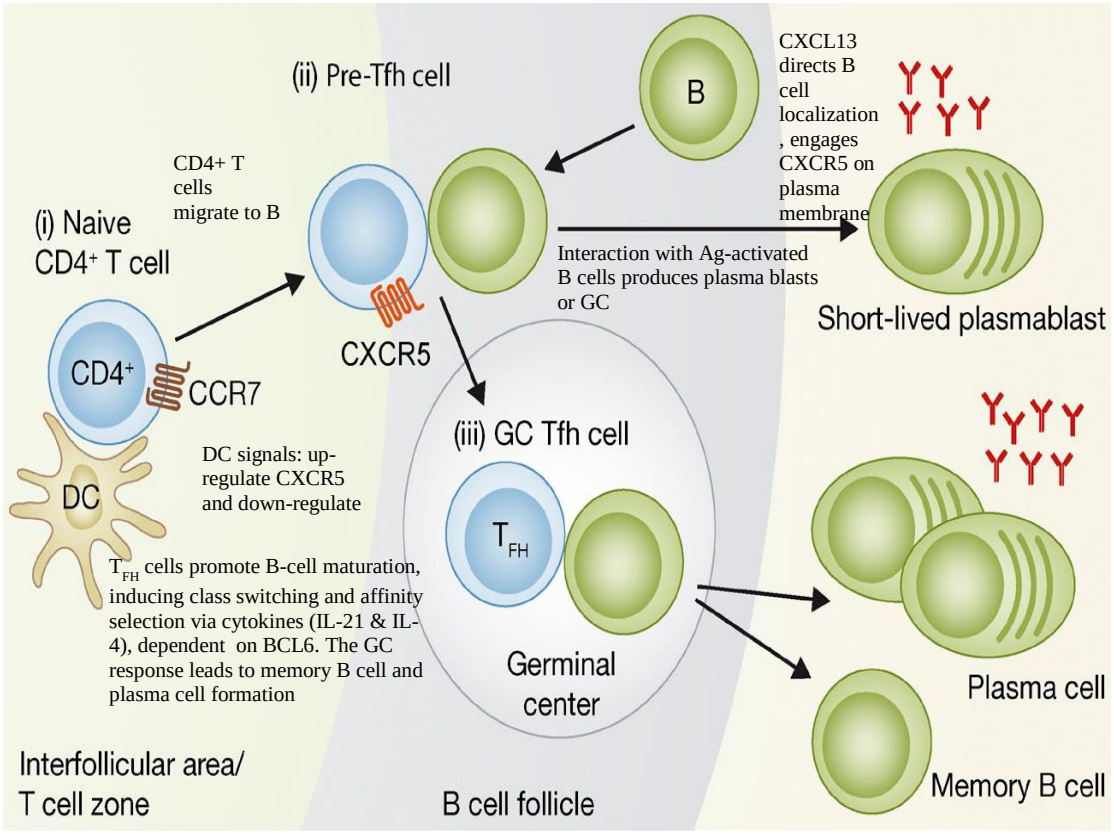

Figure 1.4 The role of T_{FH} cells in GC formation. *Adapted from (152).*

Since, T cells and specifically T_{FH} cells play a crucial role in pathogenesis of lupus *via* GC formation and generation of antibody producing plasma cells, based on our earlier findings, *we examined the role of Gr-1$^+$CD11b$^+$ MDSCs like cells on T cell independent and T cell dependent antibody responses in lupus prone (NZB x NZW) F1 mice.* The findings of this study are discussed in detail in **Chapter III (Gr1$^+$ Cells Suppress T-Dependent Antibody Responses in (NZB x NZW) F1 Male Mice through Inhibition of T Follicular Helper Cells and Germinal Center Formation; Published in *The Journal of Immunology*, 2014).**

1.6. The role of genetic factors on SLE:

Like many other systemic autoimmune diseases SLE has a very strong female bias and the role of genetics cannot be overlooked in the incidence of SLE. There are strong data from mouse and human studies which strongly point towards an important role of genetics in the incidence and progression of SLE. One of the major factors is the dosage compensation effect of the X chromosome where females (XX karyotype) and individuals affected by Klinefelter syndrome (XXY karyotype) are 10 times more susceptible to SLE as compared to males (XY karyotype) (156). The role of the X chromosome in SLE development was elegantly shown in the pristane induced lupus model using *Sry*-transgenic mice. It was shown that male castrated XX*Sry* mice were more susceptible to lupus as compared to XY^{-}*Sry* mice (157). Similarly, BXSB.*Yaa* mice containing *Yaa* (Y-linked autoimmune accelerator) which is a 4-megabase translocation of the distal end of X chromosome onto Y chromosome have exacerbated lupus like disease (158-160). One of the genes expressed on the distal end of the X chromosome is TLR7 and it has been strongly associated with susceptibility to lupus (161). Some of the most detailed insights into the genetics of SLE have come from the New Zealand Black (NZB) and New Zealand White (NZW) mixed strains (NZM). The (NZB x NZW)F1 hybrid develops a lupus like disease with a strong female bias displaying characteristic features such as - anti-nuclear antibodies and glomerulonephritis (70), while none of the parent strains develop full blown disease (162). Detailed studies lead to the identification of two separate susceptibility loci in the NZM mice, *Sle1* (located on chromosome 1) and *Sle2* (located on chromosome 4) (163,164). Congenic mice expressing either the *Sle1* or *Sle2* locus have specific symptoms but none of them develop renal disease whereas

combining *Sle1* and *Sle2* resulted in glomerulonephritis and enhanced mortality (160). This is a strong indication of the complex multigenetic nature of lupus like disease in mice. Also these studies show that the genetics involved in pathogenesis of lupus is autosomal as well as sex chromosome linked. Additionally, Pre-B cell lines from fetal livers (FLs) of lupus prone (NZB x NZW)F1 mice transferred lupus-like disease to SCID (Severe combined immuneodeficiency) mice but it is not known if the cell lines were of male or female origin (165).

It is evident that sex hormones (discussed in 1.4.2) and genetics play an important role in the incidence of lupus-like disease and its severity, but the relative contribution of each factor is not well understood. In order to address this issue we utilized a mixed BM chimera system to delineate the role of sex hormones and genetics/intrinsic hematopoietic properties of immune cells in the development of lupus like disease in (NZB x NZW)F1 mice. The results of this study are discussed in detail in **Chapter IV (Intrinsic autoimmune capacities of hematopoietic cells from female New Zealand hybrid mice; Published in *Genes and Immunity*, 2014).**

CHAPTER II

GR-1^{HIGH}CD11B^{+} CELLS SUPPRESS B CELL DIFFERENTIATION AND LUPUS LIKE DISEASE IN LUPUS PRONE MALE MICE

Abstract

Objective. Systemic lupus erythematosus (SLE) develops much more readily in females than in males. Previous research has focused primarily on identifying mechanisms pertinent to the pathology in females. The aim of the current study was to delineate active protective mechanisms in males. We present evidence of a new male-associated mechanism of protection against the development of lupus-like disease in lupus-prone

(NZB x NZW) F1 mice.

Methods. We identified previously uncharacterized cellular and functional differences in myeloid cells between male and female (NZB x NZW)F1 mice, with the use of flow cytometry, confocal imaging, *in vivo* antibody-mediated depletion, and *in vitro* cell coculture assays.

Results. A population of Gr-1^{high}Ly-6G^{+}CD11b^{+} myeloid cells was found to be constitutively increased in male (NZB x NZW)F1 mice as compared with female mice

and was regulated by testosterone. The cells were located adjacent to spleen B cell follicles *in vivo* and were found to directly inhibit cytokine-induced differentiation of naive B cells into antibody-secreting cells in vitro. Most notably, treatment with anti–Gr-1–depleting antibodies increased the spontaneous production of antinuclear autoantibodies in male (NZB x NZW)F1 mice, while a similar approach in female mice had no effect on disease development.

Conclusion. Male lupus-prone (NZB x NZW)F1 mice harbor elevated levels of a population of myeloid cells with pronounced immunosuppressive capacities that specifically target B cells and the production of antibodies in vivo. We suggest that these cells represent a male-driven inhibitory mechanism involved in the control of B cell pathogenesis, delaying (or preventing) lupus-like disease development in otherwise genetically predisposed male (NZB x NZW)F1 mice.

2.1 Introduction

Systemic lupus erythematosus (SLE) is a prototypical systemic autoimmune disorder. The disease is characterized by elevated levels of antinuclear antibodies (ANAs) and cellular infiltration of various organs, including the skin, heart, and kidney(1). Like most systemic autoimmune diseases, SLE predominantly affects women. The female-to-male ratio of newly diagnosed patients reaches 9:1 between the ages of 15 years and 45 years (66,67). This coincides with the years when female sex hormones are the most active, and thus, much research has focused on elucidating the role of female sex hormones (particularly estrogen) in SLE. For example, it is well accepted that elevated levels of estrogens affect B lymphocyte selection processes by altering the threshold for negative

selection, thereby allowing more self-reactive B cells to survive and differentiate (74,166). Testosterone, on the other hand, is believed to be protective, based on mouse studies (70,72,73,167) and clinical data from SLE patients treated with testosterone or the adrenal steroid dehydroepiandrosterone (168-170). Still, the target of regulation and the specific mechanism of protection mediated by testosterone remain unidentified.

In some animal models of SLE, female predominance is similarly observed. One such model is the (NZB X NZW) F1 hybrid mouse. Within 1 year of birth, 100% of female (NZB X NZW) F1 mice develop a lupus-like disease that is characterized by increased levels of anti-chromatin, anti-histone, and IgG anti–double-stranded DNA (anti-dsDNA) autoantibodies, as well as glomerulonephritis, IgG immune complex (IC) deposition within the kidney glomeruli followed by complement fixation, and renal disease (proteinuria) (69-71). In contrast, only ~30% of male (NZB x NZW)F1 mice develop disease within this time period. In fact, the maximum incidence in male (NZB X NZW)F1 mice reaches only ~65% (69). We used this model to pursue potent naturally occurring immunosuppressive mechanisms that actively protect genetically susceptible males against the development of lupus-like disease.

In non-autoimmune individuals, self-reactive cells are kept in check by both central and peripheral mechanisms of tolerance. The immune regulatory network consists of regulatory cells of many origins. The best known component is the regulatory T cell, which has been extensively studied both in mouse models of lupus and in SLE patients (for review, see refs. (171,172). Newer members of the regulatory family include regulatory interleukin-10 (IL-10)–producing B cells and myeloid-derived suppressor cells

(MDSCs), both of which have been studied in only a few models of lupus (92,173,174). MDSCs represent a heterogeneous pool of immature myeloid cells that are generated in the bone marrow and are released under inflammatory conditions (175,176). MDSCs have been extensively studied under tumorigenic conditions, and antibody-mediated depletion of MDSCs supports tumor eradication in tumor bearing mice. Similarly, treatment of renal cell carcinoma patients with a receptor tyrosine kinase inhibitor (sunitinib) reduces the numbers of MDSCs and diminishes tumor progression (177-179).

We report herein the identification of a population of immunosuppressive myeloid cells (Gr-1^{high}Ly- $6G^{+}CD11b^{+}$) that were significantly up-regulated in a testosterone-dependent manner in male mice. Antibody mediated depletion of the cells in male, but not female, mice resulted in elevated autoantibody production and IgG IC deposition. Accordingly, we show that (Gr-1^{high}Ly-$6G^{+}CD11b^{+}$) cells from male and female mice use different mechanisms of suppression *in vitro* and that cells from female, but not male, mice lose their suppressive capability with aging. We suggest that this population of myeloid-derived cells is heterogeneous and differentially regulated in males and females, resulting in active protection against the development of spontaneous autoimmunity in otherwise genetically predisposed males. These findings offer a significant new therapeutic target for lupus patients.

2.2 Materials and Methods

2.2.1 Mice and cells

Male and female (NZB X NZW)F1 mice (3 weeks old) were obtained from JAX Mice (The Jackson Laboratory) and were maintained at the Biological Resource Unit at Lerner

Research Institute, Cleveland Clinic. Castrated male (NZB X NZW)F1 mice (3 weeks old) were also obtained from JAX Mice. Slow-release pellets containing 12.5 mg or 25 mg of 5α-dihydrotestosterone (Innovative Research of America) were inserted subcutaneously in the neck region of castrated mice that had been anesthetized with isoflurane (Terrell; Primal Critical Care). Reconstitution was considered successful when levels of serum testosterone had equalized to the levels in age-matched unmanipulated male mice. There were no statistical differences in the levels of testosterone present in mice receiving the 12.5-mg or the 25-mg pellets. Five weeks later, mice were killed and examined. All mouse experiments were approved by the local Institutional Animal Care and Use Committee.

Antibody-treated mice were injected intraperitoneally every 3 days for 8 weeks with 500 μg of anti–Gr-1 antibody (RB6-8C5 monoclonal antibody), 500 μg of rat IgG, or sterile filtered 1X phosphate buffered saline (PBS), each in a total volume of 200 μl. For in vitro studies, Gr-1^{high}CD11b^{+}, Gr-1^{low}CD11b^{+}, and B220^{high}CD19^{high} cells were isolated by

flow-based cell sorting using a FACSAria I (BD Biosciences). All sorted cells were confirmed to be negative for CD3 and CD11c expression. Cytospin was performed using Cytology Funnel disposable sample chambers (Fisher HealthCare), Shandon Cytoslides (Thermo Scientific), and a Cytology Cytospin 2 centrifuge (Shandon). Cells were stained with hematoxylin and eosin (Newcomer Supply).

2.2.2 Flow cytometry

Flow cytometry was performed using either a FACSCalibur or a FACSAria I instrument, and all analyses were done using FlowJo version 9.5.2 software (all from BD

Biosciences). Antibodies with the following specificities were used for all analyses: CD3, CD11b, CD11c, CD19, CD21, CD23, B220 (CD45R), CD49d, F4/80, Ly-6C, CD16/32, Gr-1 (Ly-6C/6G), IgM, and IgD (all from eBioscience). Anti– Ly-6G antibody was obtained from Miltenyi Biotec and anti- CXCR2 antibody from R&D Systems.

2.2.3 Immunostaining

For histological analyses, half kidneys were harvested and fixed in 4% paraformaldehyde. Glomerular structure and inflammatory cells were determined on two 5μm sections 30μm apart after Hematoxylin/Eosin staining (Newcomer Supply). The number of mesangial cells/glomerulus was determined in a blinded fashion. For detection of IgG, IgM and complement factor 3 (C3), half kidneys were quick-frozen in OCT™ and 5μm sections were prepared and stained using TexasRed-conjugated anti-mouse IgG (Invitrogen) or anti-mouse IgM antibodies (Southern Biotech) alongside FITC-conjugated anti-mouse C3 specific antibodies (ICL, inc.). Images were collected using an HC Plan Apo 20x/0.7NA objective lens on a Leica DMR upright microscope (Leica Microsytems) equipped with a Retiga EXi Cooled CCD Camera (QImaging). Depositions were quantified using ImageProJ Plus software (Media Cybernetics) and are given as density (green or red) per area (glomerulus). Five to eight glomeruli were measured per sample and data are presented as the mean ± SEM per 8 treatment group. Detection of Gr1-expressing cells within spleens of male and female (NZB x NZW)F1 mice were done using FITC-conjugated anti-Gr1 antibody (eBiosciences), APC-conjugated anti-CD11b antibody, biotin-conjugated MOMA-1 antibody, APC or biotin-conjugated anti-B220 antibody (eBiosciences), and Alexa Fluor 568-conjugated streptavidin on 5 μm

frozen sections. Confocal images were obtained using a Leica TCS-SP-AOBS Spectral Laser Scanning Confocal Microscope (Leica Microsystems) and analyzed using Leica Confocal Software (Leica Microsystems).

2.2.4 Elution of antibodies from kidneys

Total antibodies were eluted from frozen kidneys. Briefly, kidneys were thawed, cut into smaller pieces (<1 mm diameter) and added Urea-Glycine buffer (0.5M Urea, 0.15M glycine, pH 2.8) at a 1:5 weight/volume ratio. Then samples were sonicated 5 cycles of 30 sec./30 sec. rest, and left ON at 4°C. Supernatants were harvested after centrifugation and applied to Amicon Ultra 3K centrifugal filters (EMD Millipore, MA). All kidneys were weighed before elution and the final elution volume was determined. Eluted antibodies were diluted 1:500 and tested for anti-dsDNA reactivity by ELISA as described below. The amount of antibodies per kidney is given as U/µg tissue and was calculated as: [Ab, U/µl] / [µg kidney/µl elution volume].

2.2.4 In vitro B cell differentiation

Flow-sorted $CD19^{high}$ B cells (2 x 10^5/well) were plated with variable numbers of flow sorted Gr1highCD11b+ or Gr1lowCD11b+ cells in the presence or absence of recombinant IFNα (500 units/ml, PBL InterferonSource) and recombinant CD40L (10µg/ml, EBiosciences) in complete media (RPMI1640 w/ 10% FBS, 1% non-essential amino acids, 1% pen/strep, 1mM Na-Pyruvate). After 72 hrs, cells were harvested and 25.000 cells/well were transferred onto ELISPOT plates (Millipore) pre-coated with anti-Ig antibody (Southern Biotech). Remaining cells were stained for Gr1 and B220-expressing cells and analyzed for the relative survival of each cell subset by flow

cytometry. After additional 20hrs, cells were removed and plates were developed using HRP-conjugated anti-IgG and anti-IgM specific secondary antibodies (KPL and Southern Biotech) and AEC Substrate set (BD Biosciences). The numbers of IgM or IgG-secreting cells were detected on an ELISPOT reader (CTL Immunospot) and are given as number of spots per 105 B cells. Where noted, sorted Gr1highCD11b+ cells were pretreated with 20mM N-Acetyl-L-Cysteine (NAC) a ROS inhibitor (Sigma, MO) or 10μM Nω-L-Arginine methyl ester hydrochloride (L-NAME) a NOS inhibitor (Sigma, MO) for 30 min. at 37°C, before being added to $CD19^+$ B cells.

2.2.5 Anti-nuclear Autoantibody ELISA

Serum obtained from anti-Gr1 antibody and control-treated animals, before, during and after treatment ended, was diluted 1:300 in serum diluent (sterile filtered 0.5% bovine γ-globulin, 5% gelatin, 0.05mM Tween in 1x PBS) and analyzed for levels of anti-chromatin, anti-histone and anti-dsDNA IgG autoantibodies. Briefly, microtiter plates (Immulon 2HD) were coated with purified chromatin or total histones over night at 4°C, blocked in 5% gelatin/PBS for ≥2hrs, and incubated with samples for 2 hrs. Secondary HRP-conjugated anti-mouse IgG antibodies (Invitrogen) were added for 1.5 hrs and plates were developed using 10mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) in McllWain's buffer (0.09 M Na2HPO4, 0.06 M citric acid, pH 4.6). Anti-dsDNA IgG IgG levels were determined using the manufacturer's protocol (Alpha Diagnostic International Inc., TX).

2.2.6 Cytokine ELISA

Serum cytokine levels were measured using the manufacturer's protocols (BAFF and IL-10 (EBiosciences), IL-6 (R&D Systems)). Briefly, serum samples were diluted 1:3 (BAFF), 1:10 (IL-6), or 1:4 (IL-10) and added to pre-coated Immulon 2-HB microtitre plates (Thermo Scientific, MA). Concentrations of serum cytokines were calculated based on OD values obtained from the supplied recombinant cytokine standard. All calculations were done using GraphPad Prism software (ver. 5.02).

2.2.7 Statistical analyses

The statistical significance between the means of two groups was determined using Student's t-test or the non-parametric Mann-Whitney test.

2.3. Results

2.3.1. NZB x NZW)F1 male mice harbor elevated levels of $Gr1^{high}CD11b^{+}$ cells as compared with age-matched (NZB x NZW)F1 female mice

To identify potential immune mechanisms acting differentially in males and females prior to puberty, we analyzed the cellular immune compartment present in spleens of male and female lupus-prone (NZB X NZW)F1 mice at 4 weeks of age. Contrary to our expectations, we found no significant differences between the sexes within subpopulations of T cells, B cells, and dendritic cells (Figure 2.1 A). Rather, we found consistently elevated levels of two myeloid cell populations characterized by the co-expression of CD11b (Mac-1) and Gr1 (Ly6G/Ly6C): $Gr1^{high}CD11b^{+}$ and $Gr1^{low}CD11b^{+}$ cells (Figure 2.1 A-B, $p < 0.01$). Further analyses showed that splenic $Gr1^{high}CD11b^{+}$ cells were significantly elevated in males as compared with females in percentages ($p <$

0.05-0.001), as well as in total numbers ($p < 0.05$-0.01), throughout the first 6 months of life (Figure 2.1 C, left panel). Total numbers of the corresponding population of splenic $Gr1^{low}CD11b^{+}$ cells were also significantly elevated in 4 wk old male (NZB x NZW)F1 mice as compared with females ($p < 0.01$), but not in older mice (Figure 2.1 C, right panel). To determine if the elevated levels of $Gr1^{high}CD11b^{+}$ cells in male mice were due to regulation by male sex hormone, we analyzed levels of cells in castrated and hormone-reconstituted (NZB x NZW)F1 mice. Both numbers and percentages of splenic $Gr1^{high}CD11b^{+}$ cells significantly decreased in castrated male, but not ovariectomized female, mice as compared with unmanipulated littermates ($p < 0.001$; Figure 2.1 D). The effect was specific to the effect of testosterone, as reconstitution of castrated male mice with 5α-dihydrotestosterone, which cannot be converted to estradiol, replenished levels of $Gr1^{high}CD11b^{+}$ cells. ($p < 0.0001$; Figure 2.1 D).

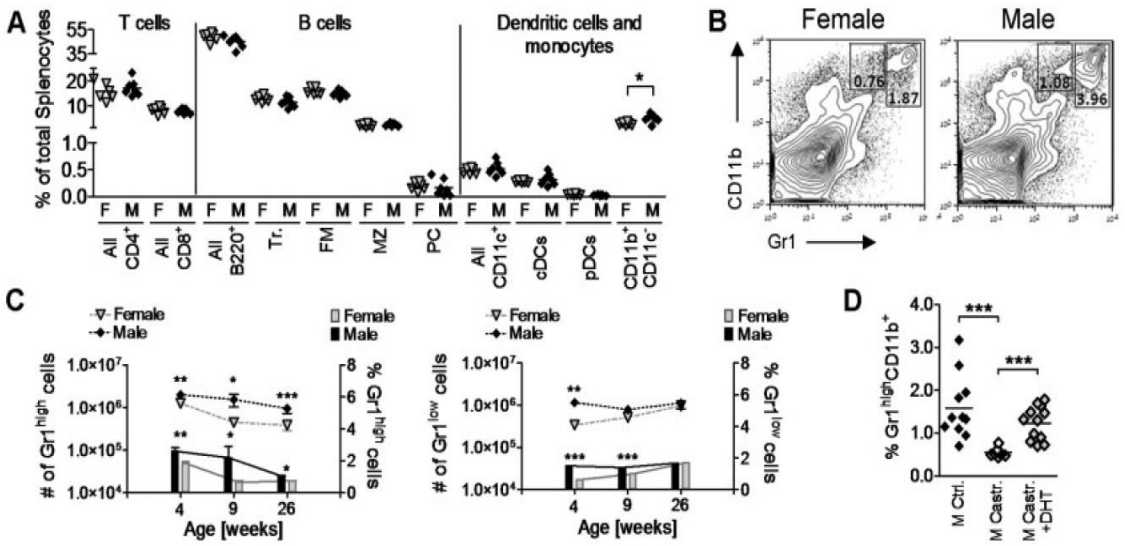

Figure 2.1 Elevated levels of splenic Gr1^{high}CD11b^{+} cells in male as compared with female (NZB x NZW)F1 mice. (A) Percentages of T cells, B cells, DCs and monocytes in 4 wk old male (M) and female (F) (NZB x NZW)F1 mice. Each data point represents an individual mouse. Tr: Transitional B cells, FM: Follicular Mature B cells, MZ: Marginal Zone B cells, PC: Plasma Cells, cDCs: conventional DCs (CD11c^{+}B220^{-}), pDCs: plasmacytoid dendritic cells (CD11c^{+}B220^{+}). **(B)** Representative contour plots showing the levels of Gr1^{high}CD11b^{+} and Gr1^{low}CD11b^{+} cells in 4 wk old male and female (NZB x NZW)F1 mice. **(C)** Absolute numbers and percentages of Gr1^{high}CD11b^{+} cells (left panel) and Gr1^{low}CD11b^{+} cells (right panel) in male (black diamonds, black bars) and female (gray triangles, gray bars) in 4, 9 and 26 week old (NZB x NZW)F1 mice. Data are shown as the average ± SEM of cells in ≥5 individual mice obtained in >5 independent analyses. **(D)** Splenic Gr1^{high}CD11b^{+} cells are regulated by testosterone. Percentage of cells was measured 5 weeks post-surgery in unmanipulated (black

diamonds), castrated (white diamonds) and castrated + 5α-dihydro-testosterone treated (gray diamonds) mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3.2. $Gr1^+CD11b^+$ cells in young (NZB x NZW)F1 mice locate to the perifollicular regions of the spleen and share surface markers with both neutrophils and MDSCs

Gr1-expressing cells have recently been found to locate to the perifollicular region in healthy humans and non-autoimmune mice (113). Confocal imaging of immunostained 4 wk old (NZB x NZW)F1 mouse spleen sections confirmed the localization of $Gr1^+CD11b^+$ cells adjacent to B lymphocytes (Figure 2.2 A). The pattern of localization was not significantly different between males and females, although we observed slightly more $Gr1^+$ cells within the marginal zone areas of males than of females (Figure 2.2 A a-c, arrows). We further characterized the cells with regard to cellular morphology and cell surface marker expression: $Gr1^{high}CD11b^+$ cells had a phenotype of $Ly6G^+Ly6C^{low}CD49d^-F4/80^-CD124^-$ and displayed a neutrophilic nuclear morphology, while Gr1lowCD11b+ cells were $Ly6G^-Ly6C^{high}CD49d^+F4/80^+CD124^{low}$ and had a monocytic appearance (Figure 2.2 B-C). These patterns are shared with, but not exclusive to, monocytic and neutrophilic MDSCs (121,175,180,181). In addition, we found that 15-25% of splenic $Gr1^{high}CD11b^+$ cells from 4 wk old (NZB x NZW)F1 male and female mice coexpressed CXCR2, the prototypic surface chemokine receptor of mature neutrophils (Figure 2.2 C, bottom panel). CXCR2 was not expressed by $Gr1^{low}CD11b^+$ cells.

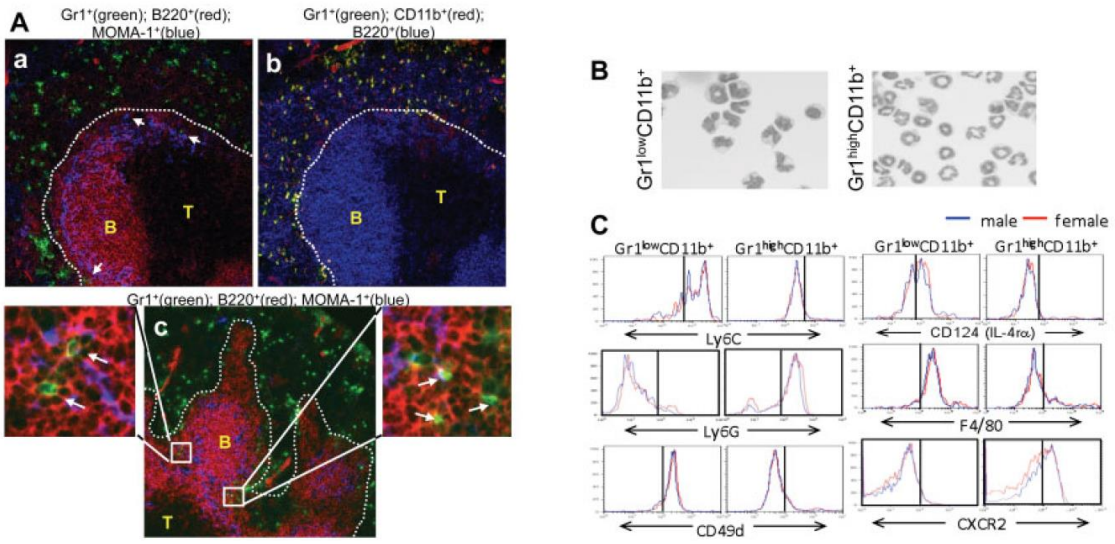

Figure 2.2 Gr1^{high}CD11b^{+} cells display phenotypic characteristics of granulocytic myeloid-derived suppressor cells. (A) Confocal microscopy analysis of the intra-splenic localization of Gr1-expressing cells in 4 week old female (a,b) or male (c) (NZB x NZW)F1 mice. Stippled lines (a and c) outlines B cell follicle and arrows (a and c) point to Gr1-expressing cells within the peri-follicular region. B: B cell follicle; T: T cell zone. Pictures were taken at 40x magnification and are representative of 5 female and 4 male 28 samples. **(B)** Cytospin analysis of Gr1^{low}CD11b^{+} and Gr1^{high}CD11b^{+} cells. Nuclear morphology was determined after H&E staining. **(C)** Flow cytometry analysis of cell surface markers on Gr1^{low}CD11b^{+} and Gr1^{high}CD11b^{+} cells from 4 wk old (NZB x NZW)F1 mice. Cells were costained for Ly6C, Ly6G, CD49d, F4/80, CD124 (IL-4rα) and CXCR2. Level of expression of each marker is given in a histogram (blue line: male; red line: female) after gating on each subset individually. Data represent 3 or more independent analyses.

2.3.3. Gr1-expressing cells suppress autoantibody production, IL-10 production, and lupus-like disease in male, but not female (NZB x NZW)F1 mice *in vivo*.

Given the well-known differential development of lupus-like disease in (NZB x NZW)F1 males and females, we investigated if the uneven levels of $Gr1^{high}CD11b^{+}$ and $Gr1^{low}CD11b^{+}$ cells in male and female mice could be involved in the control of spontaneous lupus-like disease. We treated adult 11-15 wk old male and female (NZB x NZW)F1 mice with depleting anti-Gr1 antibody every three days for 8 weeks determining disease activity by means of serum anti-nuclear autoantibody levels and proteinuria. Functional depletion was verified in PBMC fractions after 4 and 6 weeks of depletion; levels of circulating $Gr1^{+}CD11b^{+}$ cells were constitutively reduced among total PBMCs from both males (from 28% to 9.2%) and females (from 15% to 9.5%). Continuous anti-Gr1 antibody treatment of male (NZB x NZW)F1 mice resulted in significantly elevated levels of serum anti-dsDNA IgG during and after ended treatment ($p < 0.05$)(Figure 2.3 A, right panel), as well as transiently elevated anti-histone IgG levels (Figure 3A, left panel) and IgG antichromatin (data not shown). In contrast, anti-Gr1 antibody treatment of female (NZB x NZW)F1 mice had little or no effect on the level of anti-nuclear IgG autoantibodies (Figure 2.3 B).

Other serological abnormalities previously identified in female (NZB x NZW)F1 mice include elevated BAFF, IL-6 and IL-10 (109,182-184). Quantification of these factors in anti-Gr1 antibody treated mice, showed significantly elevated levels of serum IL-10 in response to the depletion of Gr1+ cells in both male and female mice (Figure 2.3 C-D), but no differences in serum levels of either BAFF or IL-6 (data not shown).

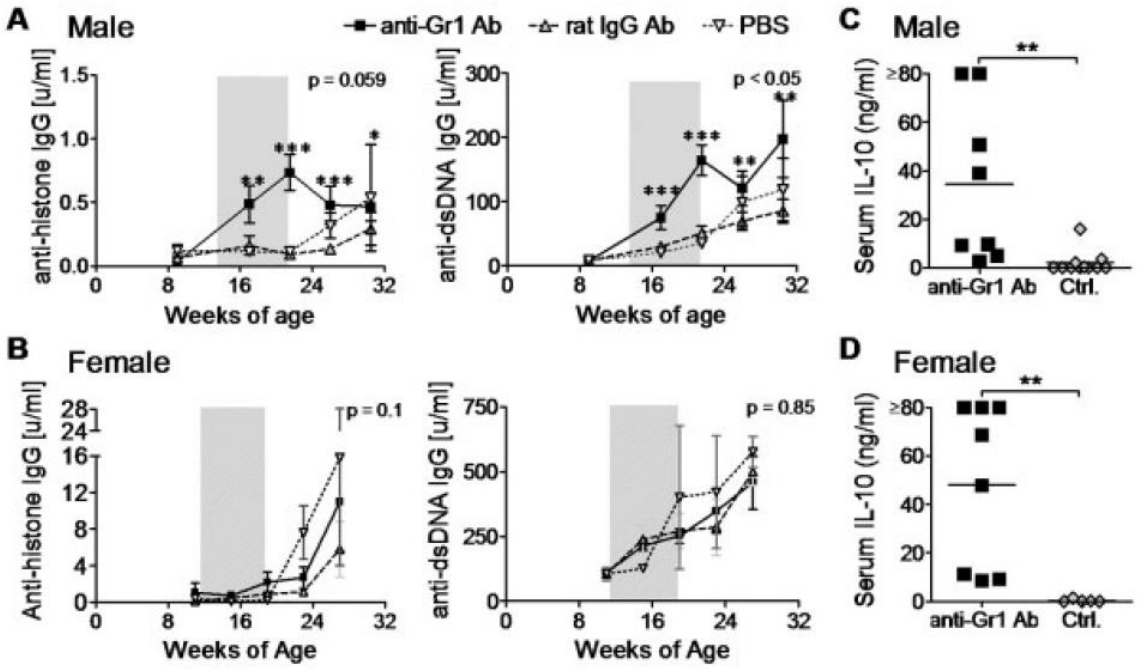

Figure 2.3 ***In vivo*** **depletion of Gr1-expressing cells increases autoantibody production and IL-10 secretion in male (NZB x NZW)F1 mice.** Male **(A)** or female **(B)** (NZB x NZW)F1 mice were treated with 500µg anti-Gr1 antibody (black squares), rat IgG antibody (gray triangles) or PBS (white triangles) every 3 days for 8 weeks (gray shaded area) starting at 11-15 weeks of age. Serum samples were obtained before, during and after treatment and levels of anti-histone IgG (left side panels) and anti-dsDNA IgG (right side panels) were determined by ELISA. The p-value for anti-Gr1 treated versus rat IgG-treated mice is given in the upper right corner of each graph. Statistical comparison of autoantibody levels at individual time points is given by asterisks (*). **(C-D)** Serum IL-10 levels were measured in male **(C)** and female **(D)** (NZB x NZW)F1 after ended treatment at 19-23 weeks of age. n = 8 (anti-Gr1 IgG treated males, females), n = 7 and 3 (rat IgG treated males, females, respectively), n = 4 and 2 (PBS-treated males, females, respectively). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3.4. The development of glomerulonephritis is unaffected by anti-Gr1 Ab treatment

Lupus-like disease in (NZB x NZW)F1 mice is also characterized by glomerulonephritis, glomerular IgG-IC deposition, and complement fixation. Anti-Gr1 antibody treated male (NZB x NZW)F1 mice displayed no significant differences in glomerular size and cellularity, or tubulointerstitial inflammation (Figure 2.4 A-B).

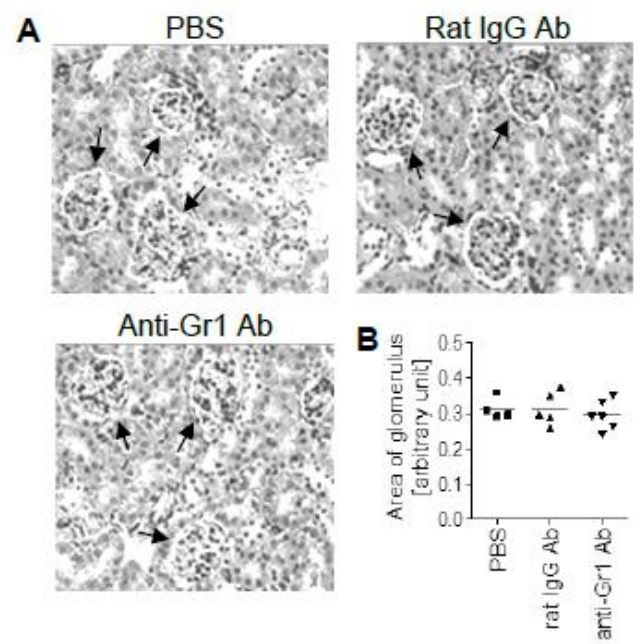

Figure 2.4 The development of glomerulonephritis is unaffected by anti-Gr1 Ab treatment. **(A)** Representative pictures of hematoxylin/eosin stained kidney sections from male (NZB x NZW)F1 mice treated with PBS (upper left), rat IgG control Ab (upper right), or rat anti-Gr1 Ab (lower left) 8 weeks post treatment. Arrows point to glomeruli. **(B)** The area per glomerulus was calculated for PBS-treated (*n* = 4 mice), rat IgG-treated (*n* = 5 mice), and anti-Gr1 Ab-treated (*n* = 6 mice). Each dot represents the average of 3-5 glomeruli per mouse.

2.3.5. IgG-IC deposition is increased in anti-Gr1 treated male mice

IgG-IC deposition was significantly elevated within the kidney glomeruli of anti-Gr1 antibody treated males, reaching levels equivalent to female age-matched control mice (Figure 2.5 A-B). This pattern was specific to IgG-IC, as no increase was observed in the levels of IgM deposition (Figure 2.5 B). Further analysis of the specificity of deposited autoantibodies after elution from a subset of the kidneys revealed elevated levels of anti-dsDNA IgG autoantibodies in anti-Gr1 antibody treated males (Figure 2.5 C). Complement fixation, as measured by C3 immunostaining, was not increased in the anti-Gr1 antibody treated group (Figure 2.5 B) and renal failure was not observed in any of the mice (dipstick proteinuria readings: PBS treated = 1.5 ± 0.0 (n = 4); rat IgG Ab-treated = 1.3 ± 0.7 (n = 5); anti-Gr1 Ab treated = 1.6 ± 0.3 (n = 7)). Thus, depletion of Gr1-expressing cells at 11-15 wks of age resulted in elevated levels of serum anti-dsDNA, anti-histone and anti-chromatin IgG, elevated serum IL-10, as well as increased anti-dsDNA IgG deposition in male mice. In female mice, depletion of Gr1-expressing cells resulted in equally elevated levels of serum IL-10, but no increase in anti-nuclear autoantibody production.

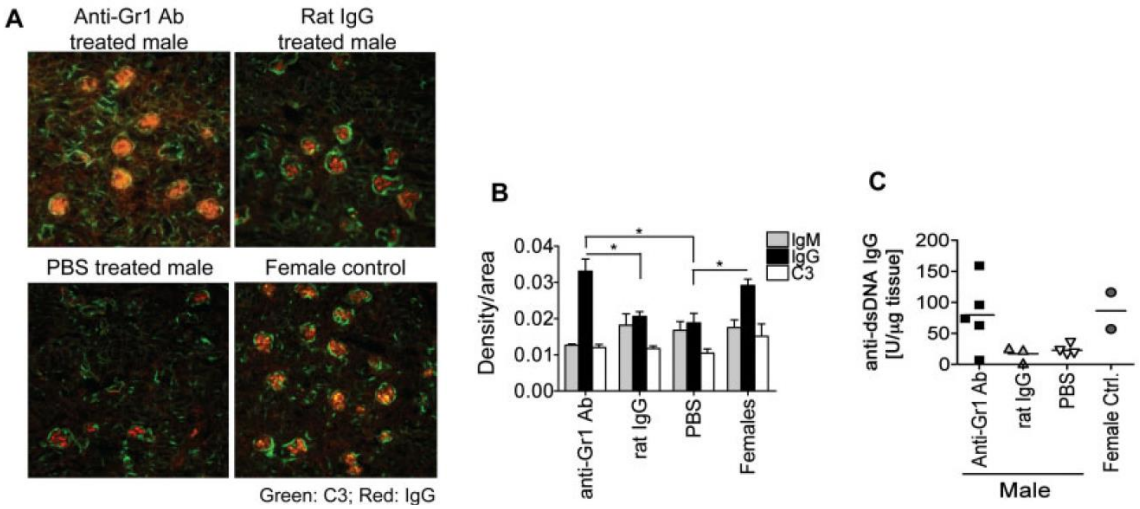

Figure 2.5 IgG-IC deposition is increased in anti-Gr1 treated male mice. (A) Representative immunofluorescence staining of IgG (red) and C3 (green) deposition in anti-Gr1 Ab, rat IgG, or PBS treated males, as well as age-matched untreated control females. Pictures shown are representative of 4-6 mice per group. **(B)** Quantification of the level of IgG, IgM and C3 staining per glomerulus as shown in **(A)**. Quantification data represent the average level of staining in individual mice ± standard error of the mean (4-6 mice per group and 5-12 glomeruli per mouse were analyzed). **(C)** Levels of dsDNA reactive IgG's were measured by ELISA after elution of total antibodies from a subset of frozen kidneys.* $p < 0.05$.

2.3.6. Gr-1^{high}CD11b^{+} cells directly suppress B cell differentiation *in vitro*

Based on the intra-splenic localization of Gr1-expressing cells and the effect on anti-nuclear autoantibody production after Gr1^{+}-cell depletion in male (NZB x NZW)F1 mice, we hypothesized that Gr1^{high}CD11b^{+} cells were involved in the control of B cell activation, differentiation and/or effector mechanisms. We therefore tested if Gr1^{high}CD11b^{+} or Gr1^{low}CD11b^{+} cells isolated from male and female 4 wk old (NZB x NZW)F1 mice could directly suppress B cell differentiation *in vitro* in a T cell-free system. Upon coculture with increasing numbers of Gr1^{high}CD11b^{+} cells, but not Gr1^{low}CD11b^{+} cells, cytokine-driven B cell differentiation was significantly inhibited in a dose dependent manner (Figure 2.6 A-B). The suppressive effect was equally present using Gr1^{high}CD11b^{+} cells from 4 wk old male or female mice.

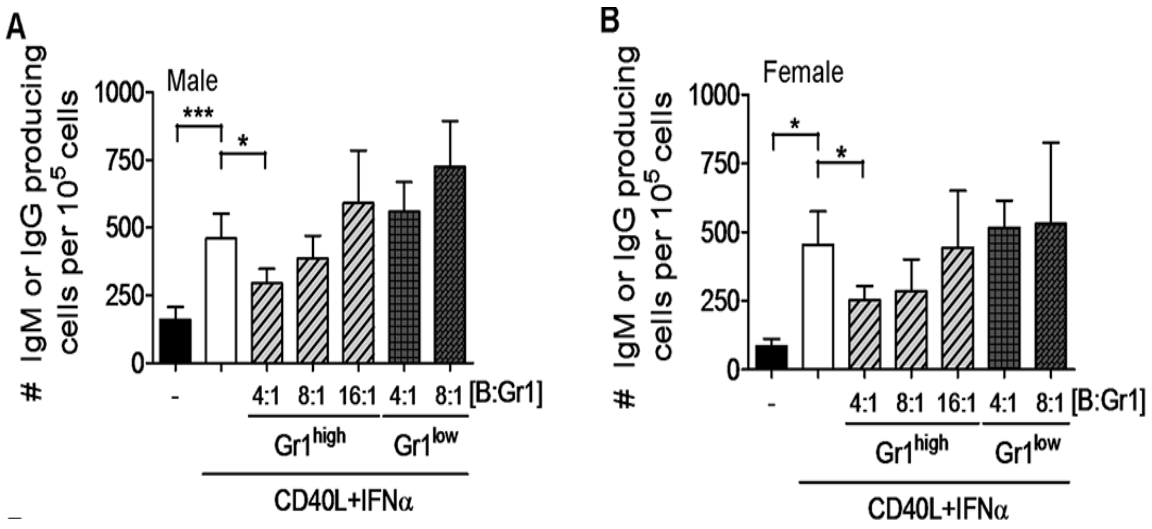

Figure 2.6 $Gr1^{high}CD11b^{+}$ cells, but not $Gr1^{low}CD11b^{+}$ cells, suppress B cell differentiation *in vitro*. B cells ($CD19^{high}$), $Gr1^{low}CD11b^{+}$ and $Gr1^{high}CD11b^{+}$ cells were isolated from spleens of 4 wk old male **(A)** or female **(B)** mice and co-cultured for 72 hrs at variable ratios (B cell to Gr1-cell ratios: 4:1, 8:1 or 16:1) in the presence of B cell differentiation-inducing cytokines (recombinant IFNα and CD40L). Total numbers of IgM- and/or IgG-secreting plasma cells were detected by ELISPOT and are given as the number of spots per 10^5 B cells. Each bar represents the mean ± SEM. Data represent 5-8 independent assays. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3.7. Gr-1^{high}CD11b^{+} cells from female, but not male, (NZB x NZW)F1 mice suppress B cell differentiation via ROS/nitric oxide (NO)

Gr1-expressing cells have been reported to suppress adaptive immune responses via numerous suppressor mechanisms including the production of reactive oxygen species (ROS) and nitric oxide (NO)(reviewed in (175)). Pretreatment of Gr1^{high}CD11b^{+} cells from *female* (NZB x NZW)F1 mice with inhibitors of either ROS or NO synthase (NOS) completely abrogated the suppressive effect of the cells (Figure 2.7 , right-side panel). In contrast, similar treatment of *male*-derived Gr1^{high}CD11b^{+} cells had no effect on the suppressive capacity of the cells (Figure 2.7, left-side panel). Thus, although male and female Gr1^{high}CD11b^{+} cells from 4 week old prepubertal (NZB x NZW)F1 mice suppress B cell differentiation equally well, the cells appear to operate through different molecular mechanisms.

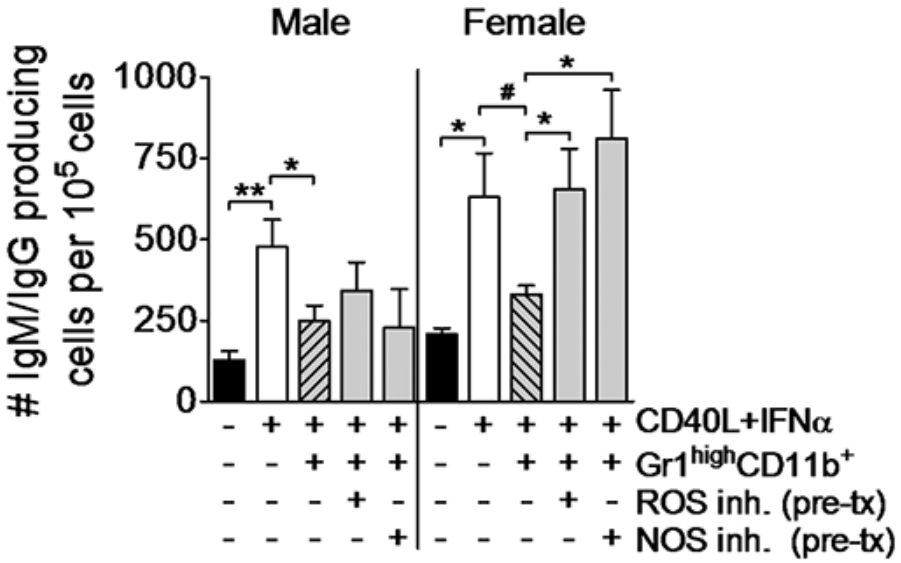

Figure 2.7 Gr-1^{high}CD11b^{+} cells from female, but not male, (NZB x NZW)F1 mice suppress B cell differentiation via ROS/nitric oxide (NO). Gr1^{high}CD11b^{+} cells were isolated from spleens of 4 wk old male or female mice and were pretreated with L-NAME (NOS inhibitor) or NAC (ROS inhibitor) before added to B cells and co-cultured with B cells as described earlier. Gr1^{high}CD11b^{+} cells each bar represents the mean ± SEM. Data represent 5-8 independent assays. * $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$.

2.3.8 Male Gr1^{high}CD11b^{+} cells remain suppressive in older mice

The *in vivo* effect of Gr1^{+}-cell depletion, as described above, was measured in older mice. We therefore analyzed the effect of Gr1^{high}CD11b^{+} and Gr1^{low}CD11b^{+} cells isolated from 14-16 wk old mice. Interestingly, *male* Gr1^{high}CD11b^{+} cells from 14-16 wk old mice remained suppressive at the 4:1 ratio [B cells : Gr1^{high}CD11b^{+} cells] ($p < 0.01$), while *female* Gr1^{high}CD11b^{+} cells lost their suppressive capacity ($p = 0.27$) (Figure 2.8 A-B). This was not due to a defect in the B cells from older (NZB x NZW)F1 females, as these cells could be suppressed using Gr1^{high}CD11b^{+} cells isolated from either 4 wk old females or 14 wk old males (Figure 2.8 B).

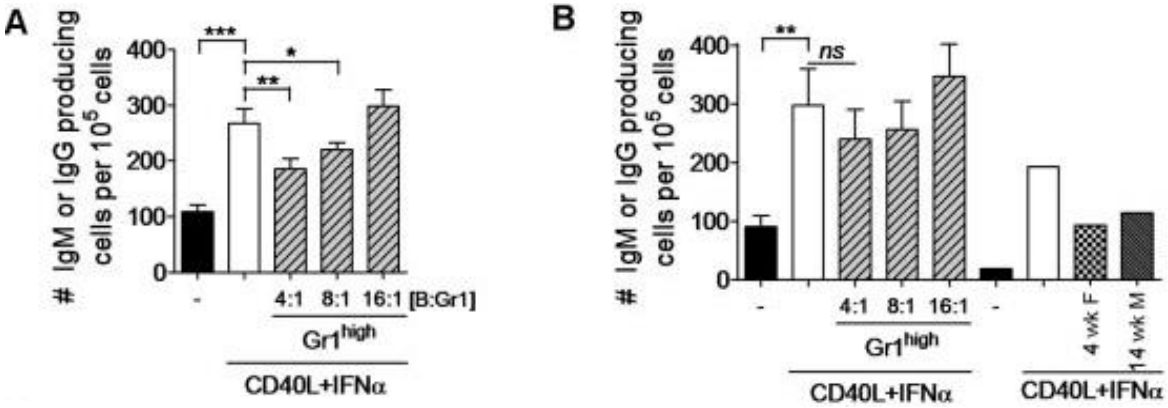

Figure 2.8 Only male Gr1^{high}CD11b^{+} cells remain suppressive in older mice. Gr1^{high}CD11b^{+} cells were flow sorted cells from 14-16 wk old male **(A)** and female **(B)** (NZB x NZW)F1 mice and added to B cell cultures in the presence of CD40L and IFNα (n = 7-8). Numbers of IgM or IgG producing plasma cells were enumerated by ELISPOT after 4 days. In **(B)** B cells isolated from 14 wk old female (NZB x NZW)F1 mice were exposed to Gr1^{high}CD11b^{+} cells from either 14 wk old females at 4:1, 8:1 or 16:1 ratios (n = 7), or from 4 wk old female or 14 wk old male (NZB x NZW)F1 mice at a 4:1 ratio (each n = 2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ns: not statistically significant.

2.3.9 $Gr1^{low}CD11b^{+}$ cells from older male and female (NZB x NZW)F1 mice stimulate B cell differentiation *in vitro*.

As female (NZB x NZW)F1 mice age, the ratio of $Gr1^{high}CD11b^{+}$ to $Gr1^{low}CD11b^{+}$ cells change in favor of the monocytic $Gr1^{low}CD11b^{+}$ population (see Figure 2.1). At the same time, anti-Gr1 antibody treatment failed to affect anti-nuclear autoantibody production in females *in vivo* (see Figure 2.3). We speculated that $Gr1^{low}CD11b^{+}$ cells in adult (NZB x NZW)F1 mice acquired a stimulatory phenotype, as previously described in both SLE patients and a pristane-induced model of lupus (113,185,186). In agreement, $Gr1^{low}CD11b^{+}$ cells from both male and female 14-16 wk old (NZB x NZW)F1 mice exhibited a stimulatory phenotype promoting B cell differentiation in our T-cell free *in vitro* cell system ($p < 0.05$) (Fig. 9 A-B).

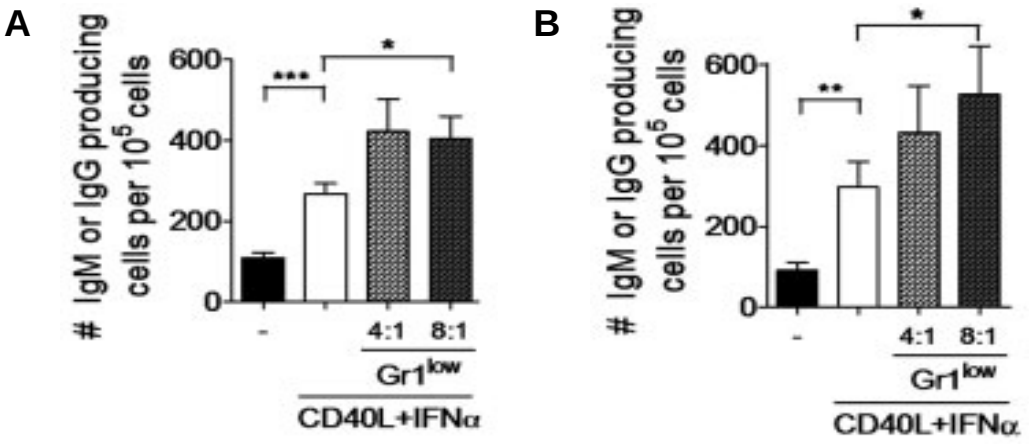

Figure 2.9 $Gr1^{low}CD11b^{+}$ cells from older male and female (NZB x NZW)F1 mice stimulate B cell differentiation *in vitro*. In older mice, $Gr1^{low}CD11b^{+}$ cells become immunostimulatory. $Gr1^{low}CD11b^{+}$ cells were flow sorted cells from 14-16 wk old male **(A)** and female **(B)** (NZB x NZW)F1 mice and added to B cell cultures in the presence of CD40L and IFNα (n = 7-8). Numbers of IgM or IgG producing plasma cells were

enumerated by ELISPOT after 4 days. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. *ns*: not statistically significant.

2.4 Discussion

SLE predominantly affects females. Over the past many years, it has been known that the male sex hormone, testosterone, exerts suppressive functions on disease development and severity (70,72,73,167-170). However, the mechanism(s) by which testosterone may influence the immune system of lupus-prone individuals in a protective manner is not well understood. In this study we investigated a mechanism of immunosuppression mediated by a population of myeloid cells ($Gr1^{high}Ly6G^{+}CD11b^{+}$), found to be present at higher levels in male than female lupus-prone (NZB x NZW)F1 mice. Manipulation of testosterone levels through pre-pubertal castration, but not estrogen levels, significantly influenced the levels of $Gr1^{high}CD11b^{+}$ cells *in vivo,* suggesting that this immunosuppressive mechanism is regulated by male sex hormone. We propose that regulation of immunosuppressive $Gr1^{high}CD11b^{+}$ cells represent one of the missing links in testosterone-mediated protection from SLE and lupus-like disease.

It is likely that $Gr1^{high}CD11b^{+}$ cells are a heterogeneous population consisting of one or more of several identified cell subsets, such as neutrophils, dendritic cell precursors and immature monocytes (175). All of these cell subsets are well-documented immunostimulatory players in the pathogenesis of established lupus as compared with healthy individuals without an autoimmune disease (101,187,188), but to our knowledge, no study has analyzed the numbers and function of the cells in genetically predisposed but undiagnosed *male versus female* individuals. Our data shows that Gr1-expressing

cells are immunosuppressive in young lupus-prone male and female mice, but that the cells remain immunosuppressive in older male mice only. Consistent with this finding, depletion of Gr-1^{high}CD11b^{+} cells in male (NZB x NZW)F1 mice resulted in increased serum ANA levels, while depletion in female mice had no significant effect. Despite increased deposition of IgG anti-dsDNA antibodies in the kidneys of male (NZB x NZW)F1 mice, they did not develop renal damage or proteinuria. The dissociation of antibody levels, IgG IC deposition, and renal disease is a well-known phenomenon that has been observed in many mouse models of SLE (189-191), suggesting that either 1) antibodies with alternate, as-yet-unknown specificities are involved in the pathogenesis of the disease or 2) additional, potentially female-intrinsic factors are required to confer susceptibility to end-organ—in this case, kidney— damage.

Gr1-expressing cells can suppress immune functions by numerous different mechanisms including ROS, NO, IL-10, TGFβ and arginase-1 (175). We do not believe IL-10 and TGFβ are involved in directly suppressing B cell differentiation, as targeting IL-10 or TGFβ by neutralizing antibodies in our *in vitro* B cell stimulation assay failed to reverse the suppressor capacity of male and female Gr1^{high}CD11b^{+} cells from male and female mice (Khan and Jorgensen, unpublished observation). In fact, removal of Gr1-expressing cells *in vivo* resulted in significantly elevated levels of IL-10, confirming a potential disease-promoting effect of this cytokine, as previously suggested in both human and animal studies (137,183). Ongoing studies aim at identifying the cellular source of this IL-10 and determining whether IL-10 is indeed involved in driving autoantibody production in anti-Gr1 antibody-depleted male (NZB x NZW)F1 mice.

In vitro, we found ROS/NO production to be critical for suppression of B cell differentiation by $Gr1^{high}CD11b^{+}$ from female (NZB x NZW)F1 mice. ROS and NO are most often associated with pro-inflammatory conditions, and levels of ROS are in fact elevated in SLE patients, especially within neutrophils, and correlated with SLEDAI scores (192,193). Similarly, elevated ROS levels have been documented in BALB/c mice with pristane-induced lupus and in the $MRL^{lpr/lpr}$ mouse model of lupus (194,195). Furthermore, inhibition of inducible NOS in female (NZB x NZW)F1 mice with proteinuria suppresses renal disease via reductions in ROS levels (196). It remains to be seen whether excessive ROS/NO production by Gr-1–expressing cells from young female mice is involved in the disease progression in older mice. As shown in our in vitro B cell differentiation assay, $Gr\text{-}1^{high}CD11b^{+}$ cells from 4-week-old male (NZB x NZW)F1 mice did not use ROS/NO as their mechanism of suppression. It is therefore possible that the heterogeneous population of $Gr\text{-}1^{high}CD11b^{+}$ cells is composed of different cell subsets in male mice as compared with female mice. Identification of the specific subset of $Gr\text{-}1^{high}CD11b^{+}$ cells with ROS/NO independent suppressor capacities in male (NZB x NZW)F1 mice will be of utmost interest in our search for new diagnostic tools and treatment strategies. These data obtained in mice lead us to suggest those differential levels of $Gr\text{-}1^{high}CD11b^{+}$ cells, as well as the potential usage of different suppressor mechanisms—if ultimately detected in humans as well—might be actively involved in delaying or preventing SLE in otherwise genetically predisposed male mice.

In addition to the $Gr1^{high}CD11b^{+}$ cells, a population of $Gr1^{low}Ly6G\text{-}CD11b^{+}$ cells is also present at higher levels in males before puberty (at 4 weeks of age), but increases in

females as they age. This population of cells concurrently acquires a stimulatory phenotype promoting cytokine-dependent B cell differentiation *in vitro*. A positive correlation between the numbers of $Gr1^{low}CD11b^{+}$ cells and autoantibody production has also previously been observed in a model of pristine-induced lupus (186), and a similar population of cells was recently found to promote T-cell independent B cell differentiation in humans (113). Whether the age-dependent accumulation of $Gr1^{low}CD11b^{+}$ cells in female (NZB x NZW)F1 mice is a result of disease progression and underlying inflammation remains to be determined.

Based on our findings reported here, we suggest that the balance between $Gr1^{high}CD11b^{+}$ and $Gr1^{low}CD11b^{+}$ cells, as well as the composition of cells within the $Gr1^{high}CD11b^{+}$ cell population, may be instrumental in protecting genetically predisposed males from lupus-like disease development. Both male and female (NZB x NZW)F1 mice display 2-3 times more $Gr1^{high}CD11b^{+}$ cells than $Gr1^{low}CD11b^{+}$ cells before puberty, and hence maintain an immunoinhibitory milieu. The ratio remains in favor of $Gr1^{high}CD11b^{+}$ cells in males up until at least 26 wks of age, however already around 9 weeks of age, female (NZB x NZW)F1 mice harbored higher numbers of $Gr1^{low}CD11b^{+}$ cells than $Gr1^{high}CD11b^{+}$ cells. Thus, as the females enter puberty the balance shifts towards the more immunostimulatory population. We therefore suggest that testosterone mediates its suppressive effect via the maintenance of a population of immunoinhibitory $Gr1^{high}CD11b^{+}$ cells. Our studies further suggest that this population is specifically involved in limiting the production of pathogenic IL-10 *in vivo*. Studies addressing ways to induce higher numbers of suppressive, ROS/NO-independent, $Gr1^{high}CD11b^{+}$ cells in

females is therefore of outmost interest as we aim to identify ways to delay or prevent B cell activation, differentiation and ultimately lupus-like disease.

Finally, it is worth mentioning, that although the majority of male mice harbor elevated numbers of $Gr1^{high}Ly6G^{+}CD11b^{+}$ cells as compared with female mice, a subset of mice (~25%) demonstrated less of an increase. This proportion correlates with the number of male (NZB x NZW)F1 mice that will develop a lupus-like disease within the first year of life (30-40%). Thus, measurements of prepubertal levels of $Gr1^{high}CD11b^{+}$ cells could potentially be used as a diagnostic tool, while manipulation of Gr1-expressing cell populations may represent a new target for immune intervention.

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CHAPTER III

GR1$^+$ CELLS SUPPRESS T DEPENDENT ANTIBODY RESPONSES IN (NZB x NZW)F1 MALE MICE THROUGH INHIBITION OF T FOLLICULAR HELPER CELLS AND GERMINAL CENTER FORMATION

Abstract

Systemic lupus erythematosus is an autoimmune disease characterized by elevated production of autoreactive Abs. The disease has a much higher prevalence in women than in men. Although testosterone has been shown to be protective in the disease, and estrogens exacerbating, the discrepancy in prevalence between men and women is still not well understood and the mechanism behind it is unknown. We have recently described that male (New Zealand black [NZB] x New Zealand white [NZW])F1 mice have higher levels of Gr1$^+$CD11b$^+$ cells, and that these cells suppress autoantibody production in vivo. In this article, we extend our findings to show that similarly to humans, female lupus-prone (NZB x NZW)F1 mice also respond with stronger Ab responses to thymus dependent Ag immunization than male littermates. Furthermore, the presence or absence of Gr1-expressing cells not only control Ag-specific Ab responses in male, but not female, (NZB x NZW)F1 mice, but also significantly alter the activation

and differentiation of CD4+ T cells in vitro and in vivo. In particular, we found that Gr1+ cells from male (NZB x NZW)F1 mice suppress the differentiation and effector function of $CXCR5^{+}PD\text{-}1^{+}$ T follicular helper cells, thereby controlling germinal center formation and plasma cell differentiation. This new finding strongly supports efforts to develop new drugs that target myeloid cell subsets in a number of T and B cell–mediated diseases with a female predominance.

3.1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoreactive Abs resulting in a variety of symptoms including fatigue, skin rashes, arthritis, and nephropathy leading to renal failure (1,197,198). One mouse model used to study lupus is the (New Zealand black [NZB] x New Zealand white [NZW]) F1 strain. Lupus-like disease in the (NZB x NZW)F1 mice is characterized by hyperactive B cells, abnormal autoantibody production, and ectopic germinal center (GC) formation, and has been shown to depend on $CD4^{+}$T cell help (127,128,199,200). Upon activation, $CD4^{+}$ T cells can differentiate into several effector cell subsets characterized by their unique production of effector cytokines, that is, Th1 (IFN-γ), Th2 (IL-4, IL-5), Th9 (IL-9), Th17 (IL-17), or T follicular helper cells (T_{FH}; IL-21). Although all of these subsets can influence B cell activation and/or differentiation, IL-21–producing T_{FH} cells are critically required for GC formation and plasma cell (PC) differentiation (154,201,202). Consistent with lupus being a B cell–dependent, autoantibody mediated disease; T_{FH} cells and IL-21 have been found to be involved in at least some aspects of lupus-like disease

development in several mouse models (203-205). Importantly, studies have also demonstrated dysregulated T_{FH} cells in SLE patients (153,206,207).

Like many other autoimmune diseases, SLE disproportionately affects women more than men (64). Hormonal therapy has been successfully applied in severe cases of SLE, suggesting that sex hormones play a major role in determining disease development in genetically predisposed individuals (168-170). Likewise, female (NZB x NZW)F1 mice develop lupus-like disease with a much higher incidence than male (NZB x NZW)F1 mice (70,71). The female predominance observed in the (NZB x NZW)F1 mouse model is known to be controlled in part by sex hormones (72,73,81,208,209). Interestingly, it has been suggested that sex hormones also partake in defining the magnitude of immune activation during infections and after immunizations in healthy individuals ((209) and reviewed in Ref. (210)).

Our laboratory recently described the presence of a population of testosterone-regulated immunosuppressive $Gr1^+CD11b^+$ cells in male (NZB x NZW)F1 mice (211). Particularly, we found that depletion of Gr1-expressing cells augmented spontaneous autoantibody production in male (NZB x NZW)F1 mice, whereas a similar strategy had no effect on autoantibody production in female mice. In this study, we tested whether the immune-suppressive function of $Gr1^+CD11b^+$ cells observed in male lupus-prone (NZB x NZW)F1 mice also affected immune activation in response to exogenous Ag. As expected, male (NZB x NZW)F1 mice responded less vigorously to immunization with a T-dependent Ag, whereas depletion of Gr1-expressing cells augmented the Ag-dependent

Ab response in males, but not in females. In correlation, numbers of both T_{FH} cells and GC B cells were elevated after $Gr1^+$ cell depletion. Thus, manipulation of analogous human cells might offer a new potential target for improving vaccination efficacy in individuals with low Ab responses, as well as repressing Ab production in systemic autoimmunity.

3.2 Materials and Methods

3.2.1 Mice and Immunizations

Three-week-old male and female (NZB x NZW)F1 mice were obtained from The Jackson Laboratories and kept at the Biological Resource Unit at Lerner Research Institute, Cleveland Clinic. All mouse experiments were approved by the local Institutional Animal Care and Use Committee. Mice were bled at 8 wk of age, and PBMCs were analyzed for the presence of $Gr1^+CD11b^+$ cells by flow cytometry, allowing for stratification of the mice into equal groups based on their basal levels of $Gr1^+CD11b^+$ cells. At 9 wk of age, mice were injected i.p. with 20 μg (4-hydroxy-3-nitrophenylacetyl)$_{27}$ conjugated chicken γ-globulin (NP_{27}-CGG) or NP_{27}-FICOLL [both from Biosearch Technologies, Petaluma, CA]), in a ratio of 1:1 with CFA (Sigma-Aldrich, St. Louis, MO), or with 1x PBS. Each injection was done in a total volume of 200 μl. Ab-treated mice were additionally injected i.p. every 3 d starting on day 21 with either 500 μg rat anti-mouse Gr1 (clone RB6-8C5, a kind gift from Dr. Pearlman, Case Western Reserve University, Cleveland, OH), 500 μg control rat IgG, or sterile-filtered PBS, each in a total volume of 200 μl as previously described (211). This treatment strategy was based on data showing that Gr1-expressing cells were fully depleted from BM, spleen, and blood for 2 d, and only began to reappear

on day 3 after injection with anti-Gr1 Ab (data not shown). Ab treatment was continued for up to 28 d as stated in the text. Mice were bled for serum by tail-vein bleeding on day -1, day 7, and every 7 d thereafter as applicable.

3.2.2 Flow cytometry

Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA), and all analyses were done using FlowJo Version 9.3. Abs with the following specificities were used for all analyses: FITC-conjugated anti- CD3 (clone 145-2C11), PE- or allophycocyanin-conjugated anti-CD4 (clone L3T4), PerCP-conjugated anti-CD8 (clone 53-6.7), PE- or allophycocyanin-conjugated anti-CD11b (clone M1/70), PE-conjugated anti-CD38 (clone 90), FITC- or PerCP-conjugated anti-B220 (CD45R; clone RA3-6B2), FITC-conjugated anti-CD62L (clone MEL-14), PerCP-conjugated anti-Gr1 (Ly6G/6C; clone RB6-8C5), biotin-conjugated anti–GL-7 (clone GL-7), PE-conjugated anti-CD278 (ICOS; clone 7E.17G9), PE-conjugated anti-CD279 (PD-1; clone MIH4), and PE-conjugated streptavidin (all from eBioscience, San Diego, CA). PE-conjugated anti-CD138 (clone 281-2) and biotin-conjugated anti-CXCR5 (clone 2G8) Abs were purchased from BD Biosciences. All flow-based analyses were based on the gating of live cells as given by forward and side scatter properties.

3.2.3 ELISA

Serum was obtained from NP_{27}-CGG immunized anti-Gr1 Ab and control Ab-treated animals before, during, and after treatment. 2HB (Immulon) 96-well microtiter plates were coated with 5 μg/well NP5 conjugated to BSA in PBS overnight and blocked for 2 h at room temperature with 5% gelatin in PBS. Serum was diluted 1:10,000 in serum

diluent (5 μg/ml bovine γ-globulin, 5% gelatin, 0.05% Tween in PBS). A total of 100 μl/ well diluted serum was plated and incubated for 2 h at room temperature. All washes were done using 0.05% Tween in PBS. Plates were developed using HRP-conjugated anti-IgG1 secondary Abs and 10 μg/ml 2,29-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) in Mcilwain's buffer (0.09 M Na2HPO4, 0.06 M citric acid, pH 4.6), and colorimetric readings were done at 405 nm on a Victor 3 plate reader (Perkin Elmer, Waltham, MA). All samples per experiment were run at the same time, allowing for direct comparisons of OD values to determine Ab levels between samples.

3.2.4 T cell proliferation assay

Spleen single cells were prepared from 9-wk-old male and female (NZB xNZW)F1 mice. $Gr1^{high}CD11b^{+}$ and $Gr1^{low}CD11b^{+}$ cells were sorted by high-speed cell sorting (FACSAria II; BD Biosciences). Total T cells were isolated from female (NZB x NZW)F1 age-matched mice by MACS separation using CD90.2 microbeads (Miltenyi Biotech, CA). After isolation, T cells were labeled with CFSE (Sigma-Aldrich). After labeling, cells were washed and reconstituted in cell culture media (RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin, 1 μM Na-pyruvate, 1xMEM). A total of 2 x 10^5 T cells/well were plated in 96-well microtiter plates in the presence or absence of plate-bound anti-CD3 and anti-CD28 Abs (10 μg/ml each; both from eBioscience). Where noted, 5 x10^4 flow-sorted $Gr1^{high}CD11b^{+}$ or $GR1^{low}$ $CD11b^{+}$ cells were added to the cultures at time 0 h. After 48 hr, cells were harvested and stained with allophycocyanin-conjugated anti- CD4 and PerCP-conjugated anti-CD8 Abs (eBioscience), allowing for separate analysis of proliferation of $CD4^{+}$ and $CD8^{+}$ T cell subsets.

3.2.5 T_{FH} cell differentiation assay

Naive CD90.2+CD62Lhigh T cells and $Gr1^+CD11b^+$ cells were sorted by high-speed cell sorting (FACSAriaII; BD Biosciences) from spleens of 9-wk-old male (NZB x NZW)F1 mice. A total of 3 x 10^5 naive T cells was plated in the presence or absence of $Gr1^+CD11b^+$ cells at different ratios ($Gr1^+$/T cell ratio = 1:4, 1:8, and 1:16). Cells were cultured in cell culture media (RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin, 1 μM Napyruvate, 1x MEM) in the presence of plate-bound anti-CD3 and anti- CD28 Abs (3 μg/ml each), 10 μg/ml anti–IL-4 Ab (eBioscience), 10 μg/ml anti–IFN-γ Ab (eBioscience), 20 μg/ml anti–TGF-b Ab (R&D Systems, Minneapolis, MN), 50 ng/ml rIL-21, and 100 ng/ml IL-6 (both from Biolegend, San Diego, CA) (29). The media were replaced with fresh media containing the same amounts of cytokines on day 3. After 5 d, cells were harvested and stained using the following Abs and conjugates: anti–PD-1 (FITC), biotinylated anti-CXCR5 and streptavidin-PE conjugate, anti-Gr1 (PerCP), and anti-CD4 (allophycocyanin; all from eBioscience). TFH cells were identified as $CD4^+Gr1^-CXCR5^+PD\text{-}1^+$ by flow cytometry as previously described (212).

3.2.6 Immunofluorescence staining

Two-millimeter slices of spleens were taken from mice on days 3, 5, 7, and 14 postimmunization and quick-frozen horizontally in OCT (Tissue-Tek, Sakura, CA). Five-micrometer sections were prepared and stained with biotinylated anti–GL-7/streptavidin-conjugated allophycocyanin and Pacific Blue–conjugated anti-B220 (all from eBioscience). Images were collected using an HC Plan Apo 103/0.7NA objective lens on

a Leica DMR upright microscope (Leica Microsystems) equipped with a Retiga EXi Cooled CCD Camera (QImaging). Numbers of GCs were counted from three distinct fields per spleen and the average number was used for statistical analyses.

3.2.7 Statistical analyses

All statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, CA; http://www.graphpad. com). Statistical significance between the means of two groups was determined using Student t test or the nonparametric Mann–Whitney U test as applicable. Analyses of in vivo kinetics of TFH and GC B cells were done by two-way ANOVA tests. Analysis of Gr1+ cell– mediated suppression of TFH differentiation in vitro was done using the one-way ANOVA test.

3.3. Results

3.3.1 Female (NZB x NZW)F1 mice produce more Ab in response to immunization than male (NZB x NZW)F1 mice

To determine whether male and female (NZB x NZW)F1 mice responded differently to Ag challenge, 9-wk-old mice were immunized i.p. with NP-CGG/CFA known to elicit a strong T-dependent IgG_1 response (213). Four weeks post-immunization (day 28), female (NZB x NZW)F1 mice produced significantly higher titers of anti-NP–specific IgG1 Abs ($p < 0.001$) than male (NZB x NZW)F1 mice (Figure 3.1A). In a separate study, we determined the numbers of splenic GC B cells and PCs in male and female (NZB x NZW)F1 mice 2 wk post-immunization (day 14). Female (NZB x NZW)F1 mice expressed significantly elevated numbers of splenic GL-7^+B220^+CD38^{low} GC B cells ($p <$

0.05) and $CD138^{+}B220^{low}$ IgM^{low} PCs ($p < 0.05$) as compared with male (NZB x NZW)F1 mice (Figure 3.1B, 3.1C).

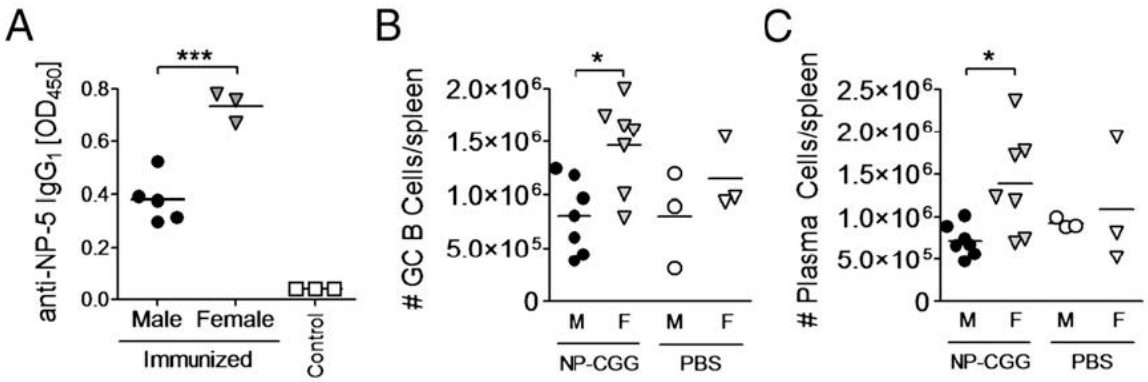

Figure 3.1 Female (NZB x NZW)F1 mice have stronger Ab responses than males in response to T dependent Ag. (A) Male and female (NZB x NZW) F1 mice were immunized at day 0 with 20 µg NP_{27}- CGG in CFA or control PBS. **(A)** On day 28, mice were analyzed for serum anti-NP5 IgG1 Abs by ELISA. ***$p < 0.001$, Student *t* test. **(B** and **C)** A separate cohort of mice were immunized as in **(A)** and euthanized on day 14. Total number of splenic $GL7^{+}$ GC B cells and $CD138^{+}B220^{low}$ PCs were enumerated. Each symbol represents one mouse. *$p < 0.05$, Mann–Whitney *U* test.

3.3.2. $Gr1^+$ cells suppress Ab responses to T-dependent Ag challenge in male but not female (NZB x NZW) F1 mice

We have previously shown that male (NZB x NZW) F1 mice have constitutively higher levels of $Gr1^+CD11b^+$ cells, and that depletion of $Gr1^+$ cells *in vivo* reduces the levels of antinuclear Abs in male but not female (NZB x NZW)F1 mice (211). To determine whether $Gr1^+$ cells play a similar role in the NP-CGG/CFA–induced Ab response, we treated male and female (NZB x NZW)F1 mice with anti-Gr1–depleting Abs or control rat IgG Abs continuously every 3 d starting 1 d before immunization. Treatment with anti-Gr1–depleting Ab did not significantly affect other cell populations **(Figure 3.2A)**. As expected, depletion of Gr1- expressing cells elevated the anti-NP–specific IgG1 Ab titers in male mice as compared with control rat IgG-treated males ($p < 0.001$; Figure 3.2B), whereas depletion of Gr1-expressing cells had no significant effect on the Ab response in female mice ($p = 0.17$; Figure 3.2C). When analyzing the splenic compartment at 2 wk post-immunization we found that depletion of Gr1-expressing cells resulted in higher numbers of both GC B cells and PCs in male mice ($p < 0.05$), but again with no effect in female mice (Figure 3.2D, 3.2E). Notably, consistent with their autoimmune phenotype, unimmunized female (NZB x NZW)F1 mice expressed continuously increased numbers of PCs as compared with male (NZB x NZW)F1 mice ($p < 0.05$; Figure 3.2E). Thus, Gr1-expressing cells suppress immunization-induced Ab responses in male, but not female, mice.

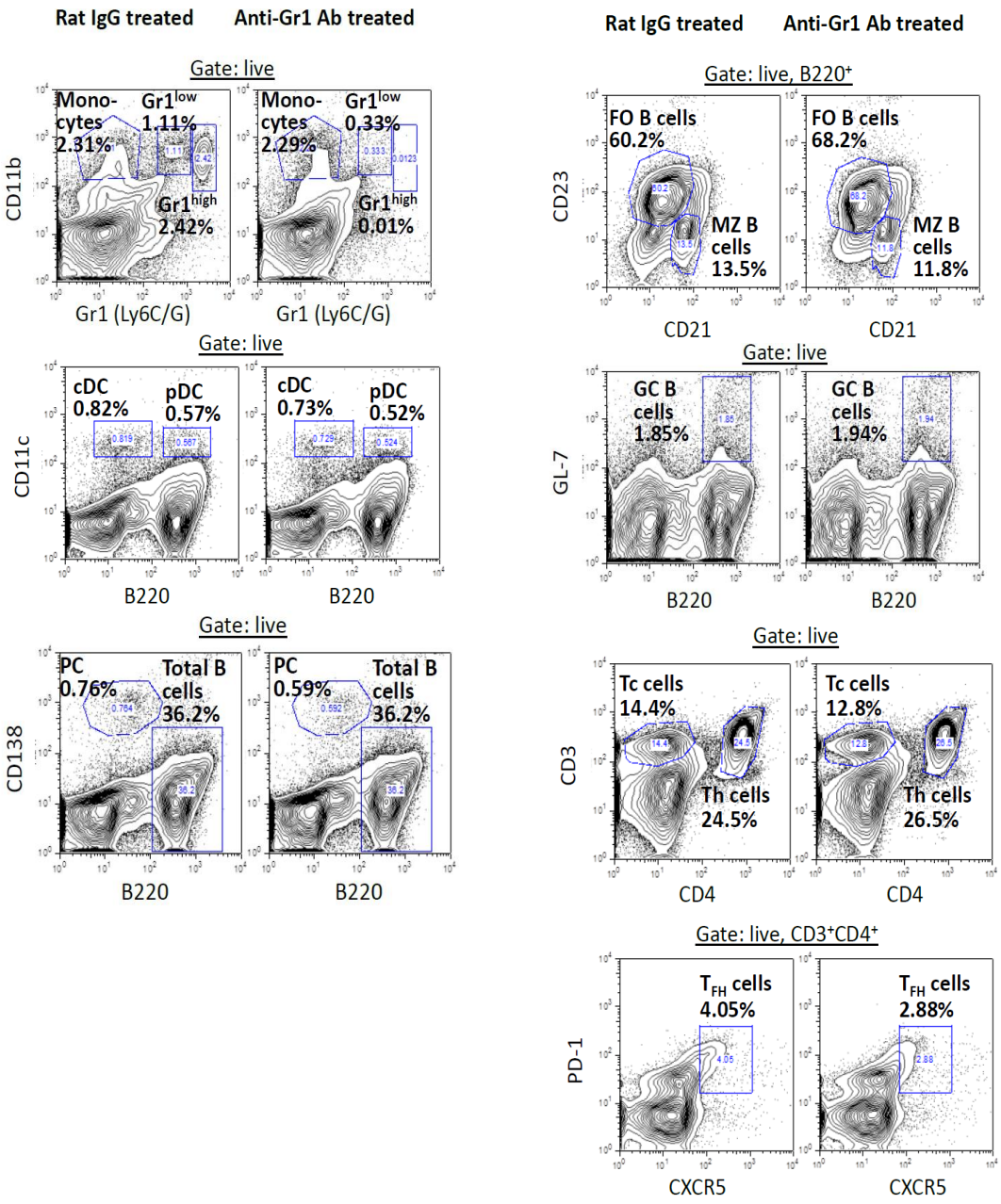

Figure 3.2(A) Treatment with anti-Gr1–depleting Ab did not significantly affect other cell populations. Injection of Anti-Gr1 depletion antibody does not affect non-Gr1- expressing cell subsets in the spleen. Male (NZB x NZW)F1 mice were injected with 500 µg anti-Gr1 antibody (column 2 and 4) or 500 µg rat IgG control antibody (column 1 and 3). After 48 hours, spleens were harvested and the relative levels of lymphoid- and myeloid derived cell subsets were defined by flow cytometry.

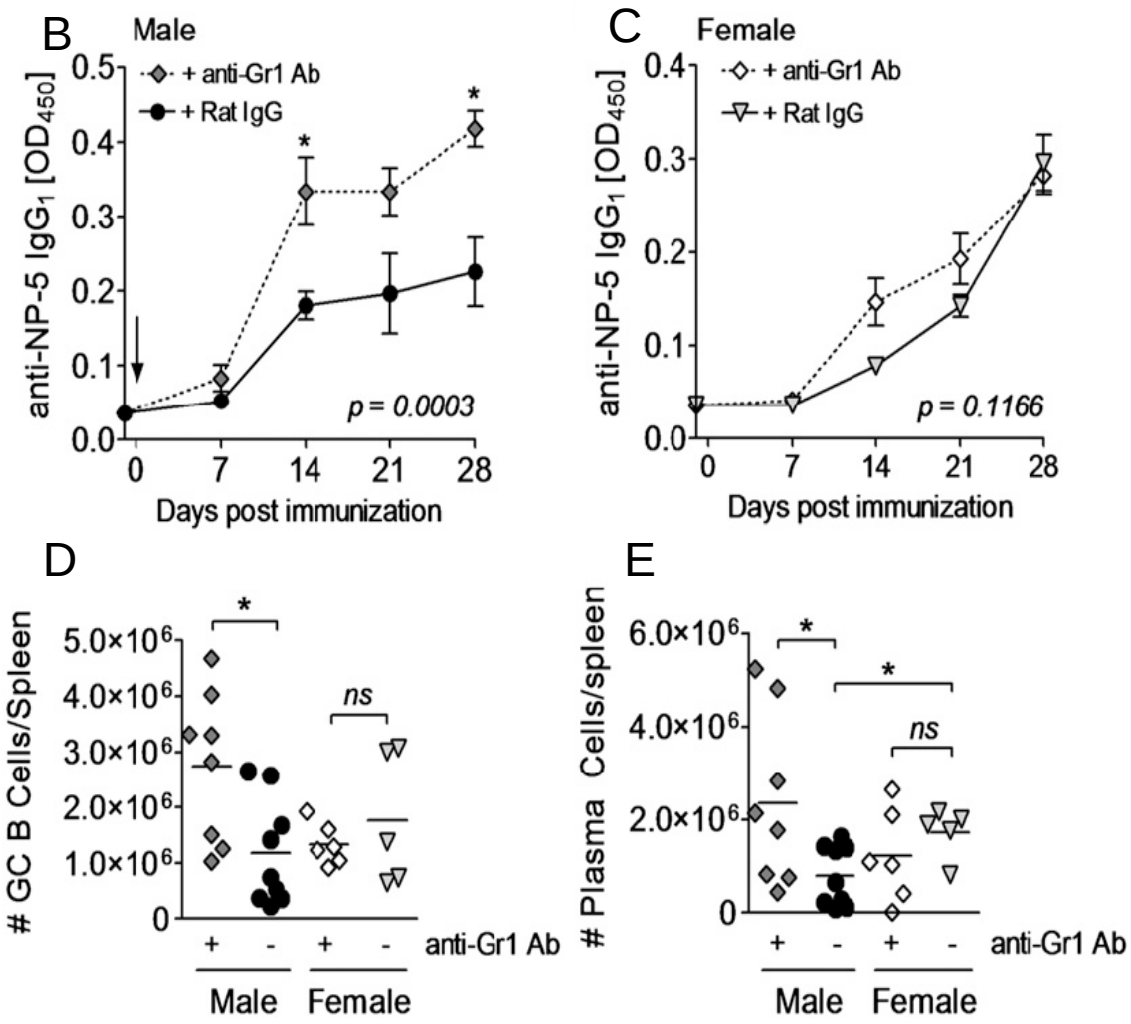

Figure 3.2(B-E) Depletion of Gr1$^+$ cells augments Ab responses after TD-Ag immunization. Male and female (NZB x NZW)F1 mice were immunized at day 0 with 20 μg NP_{27}-CGG in CFA. Where indicated, mice were additionally treated with an anti-Gr1–depleting Ab or control rat IgG every 3 d starting at day -1. Serum from males **(B)** and females **(C)** was collected every 7 d and tested for levels of anti-NP_5 IgG_1 Abs by ELISA. An asterisk above the graph indicates statistical difference at a single time point

(Student *t* test), whereas the *p* value inserted at the lower right of the graph denotes the statistical difference between anti-Gr1 Ab and control IgG–treated mice over time (two-way ANOVA test). **(D** and **E)** A separate cohort of mice were immunized and treated as in **(B)** and **(C)**, but sacrificed at day 14 post-immunization. Splenic GC B cells **(D)** and PCs **(E)** were assessed by flow cytometry. n = 5–9/ treatment group. *$p < 0.05$, Mann–Whitney *U* test.

3.3.3 Gr1$^+$ cells suppress Ab responses only to T-dependent Ag challenge in male (NZB x NZW) F1 mice

To determine whether the suppressive effect was dependent on T cells, an additional cohort of male (NZB x NZW)F1 mice was immunized with NP-Ficoll, a T-independent Ag, and treated with either depleting anti-Gr1 Ab or rat IgG control Ab. Depletion of Gr1-expressing cells had no effect on the Ab response and B cell differentiation induced after T-independent Ag immunization (Figure 3.3A). Also there was no difference in the numbers of GC B cells and PCs between anti-Gr1 Ab or rat IgG control Ab treated animals (Figure 3.3B, 3.3C). Thus, the in vivo suppressive effect of Gr1+ cells is dependent on T cells.

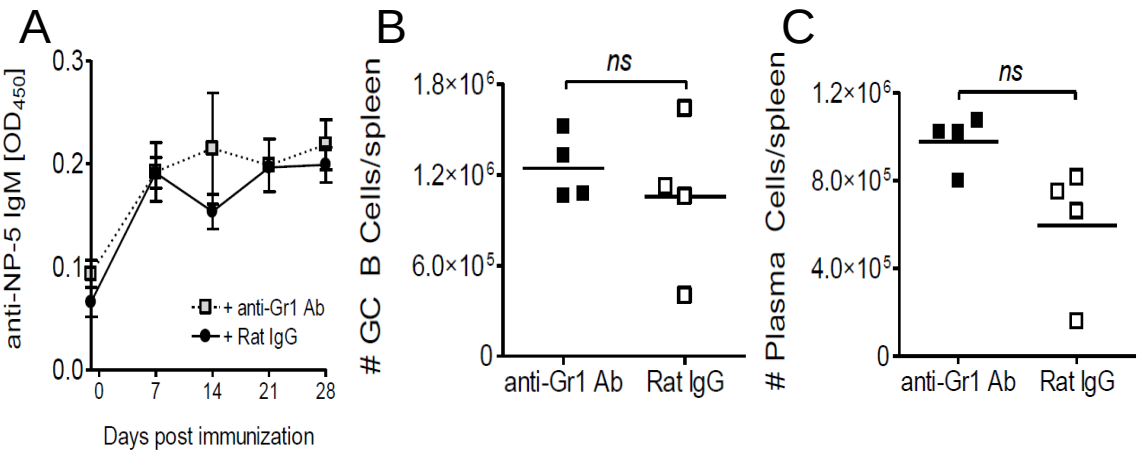

Figure 3.3 The T-independent immune response in not regulated by Gr1$^+$ cells in male (NZB x NZW)F1 mice. Male (NZB x NZW)F1 mice were immunized with NP_{27}-FICOLL/CFA on day 0. Mice were treated with depleting anti-Gr1 Ab (*n* = 4) or control rat IgG (*n* = 4) every three days starting on day -1. Serum was obtained every 7 days and tested for anti-NP antibodies **(A)**. Total numbers of GC B cells **(B)** and PCs **(C)** were enumerated from spleens of treated mice upon sacrifice on day 28.

3.3.4 Male, but not female, $Gr1^{low}$ $CD11b^{+}$ cells suppress T cell proliferation *in vitro*

Gr1-expressing cells consist of two major cell subsets expressing high and low levels of Gr1, respectively. Both subsets have been shown to have suppressive functions in cancer studies (reviewed in Ref. (175)); however, $Gr1^{low}$Ly6C+CD11b+ cells have been specifically associated with T cell suppression in another model of lupus (214). We hypothesized that $Gr1^{low}CD11b^{+}$ cells from male (NZB x NZW)F1 mice could have a similar suppressive effect on T cells and hence be involved in the in vivo effect of $Gr1^{+}$ cells during T-dependent Ab responses. $Gr1^{high}CD11b^{+}$ and $Gr1^{low}$ $CD11b^{+}$ cells were sort purified from male and female 4-wk-old (NZB x NZW)F1 mice (Figure 3.4A) and added to CFSE-labeled MACS-purified T cells in the presence or absence of anti-CD3 and anti-CD28 cross-linking Abs. $Gr1^{low}CD11b^{+}$ cells, but not $Gr1^{high}$ $CD11b^{+}$ cells, suppressed T cell proliferation in vitro (Figure 3.4B, 3.4C). Although both male- and female-derived $Gr1^{low}CD11b^{+}$ cells from 4-wk-old mice functionally suppressed T cell proliferation, only suppression by male-derived cells reached statistical significance (male: $p < 0.01$; female: $p = 0.056$; Figure 3.4D). This difference became even more apparent when we compared the suppressive effect of male and female $Gr1^{low}CD11b^{+}$ cells obtained from 9-wk-old (NZB x NZW)F1 mice (Figure 3.4E), and is consistent with the differential effect of in vivo $Gr1^{+}$ cell depletion in 9-wkold male and female (NZB x NZW)F1 mice as described earlier (Figure 3.2).

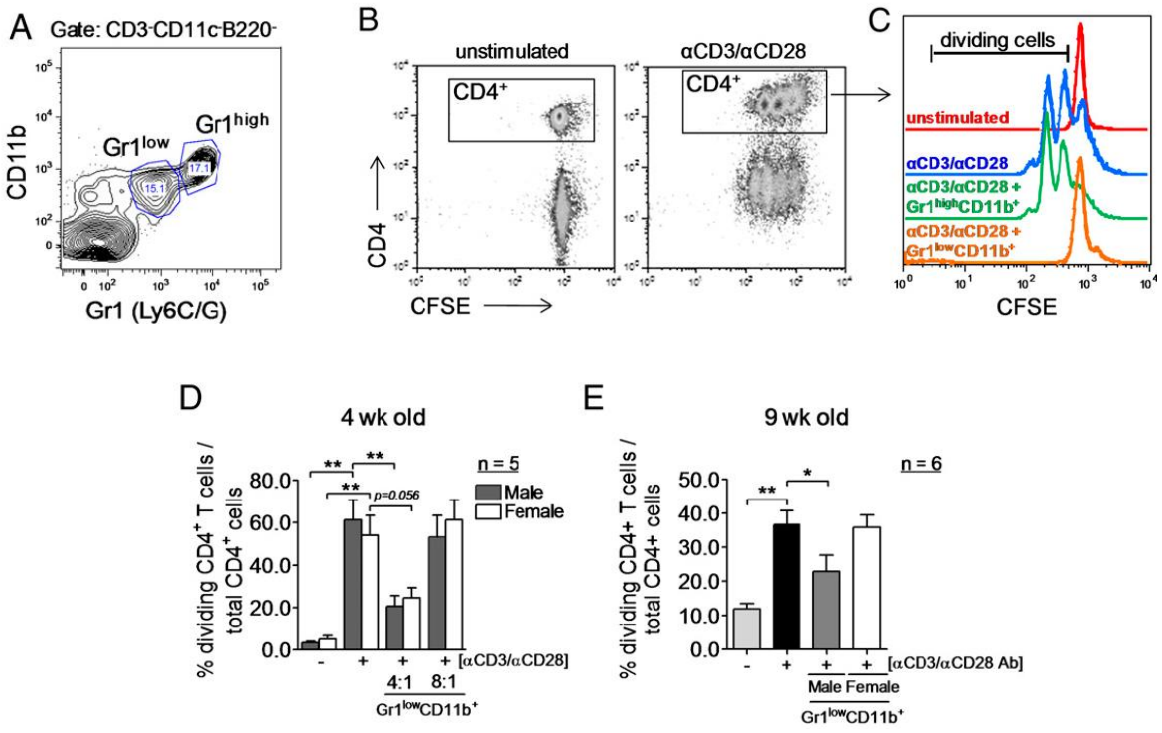

Figure 3.4 Male, but not female, Gr1^{low}CD11b^{+} cells from 9-wk-old (NZB x NZW)F1 suppress T cell proliferation *in vitro*. (A) Gating strategy for flow cytometry–based isolation of Gr1^{high}CD11b^{+} and Gr1^{low}CD11b^{+} cells. Both populations of cells reached >99% purity after isolation. **(B)** The identification of CFSE-labeled CD4^{+} T cells after 2 d of culture either unstimulated (none) or stimulated (anti-CD3/CD28). **(C)** Histogram plots showing the percentage of CD4^{+} cells that underwent >1 divisions in culture (gate: dividing cells) in the presence or absence of anti-CD3/anti-CD28 stimulation and Gr1^{high} CD11b^{+} or Gr1^{low}CD11b^{+} cells (4:1 ratio of T cells to Gr1^{+} cells). **(D)** The average of five independent experiments (mean ± SEM) using cells from 4-wkold mice is shown. $^{**}p <$ 0.01, paired Student *t* test. **(E)** Gr1^{low}CD11b^{+} cells from male (NZB x NZW)F1 mice remain suppressive in 9-wk-old mice, whereas female-derived Gr1^{low}CD11b^{+} cells lose

their capacity to suppress. Experiments were done as shown in **(A)–(D)**. Bars show the mean ± SEM of six independent analyses. *$p < 0.05$, **$p < 0.01$, paired Student *t* test.

3.3.5 Depletion of Gr1-expressing cells during immunization of male (NZB x NZW)F1 mice alters the distribution of $CD4^+$ effector T cell subsets

Because the suppressive effect of Gr1-expressing cells after immunization is dependent on T cells, and $Gr1^{low}CD11b^+$ cells specifically suppress T cell proliferation in vitro, we expected T cells to be differentially expressed in immunized male (NZB x NZW)F1 mice treated with anti-Gr1–depleting Ab or control rat IgG. Contrary to our prediction, total numbers of both $CD4^+$ and $CD8^+$ T cells were unaffected by the absence of $Gr1^+$ cells (Figure 3.5A, 3.5B). Further analyses showed that the percentage of $CD4^+$ T cells expressing markers consistent with memory-effector T cells ($CD44^{high}CD62L^{low}$), T_{FH} cells ($ICOS^+PD\text{-}1^+CD4^+$) were significantly increased in the absence of Gr1-expressing cells ($p < 0.001$, $p < 0.01$, respectively; Figure 3.5C). T regulatory cells ($Foxp3^+CD4^+CD25^+$) were also increased in $Gr1^+$ cell–depleted mice as compared to non-depleted mice, but the difference failed to reach statistical significance ($p = 0.056$). In contrast, the percentage of naive $CD4^+$ T cells ($CD44^{low}$ $CD62L^{high}$) significantly decreased ($p < 0.01$) after $Gr1^+$ cell depletion (Figure 3.5C). Thus, $Gr1^+$ cells appear to regulate differentiation of $CD4^+$ T cells during thymus-dependent (TD) Ab responses *in vivo*.

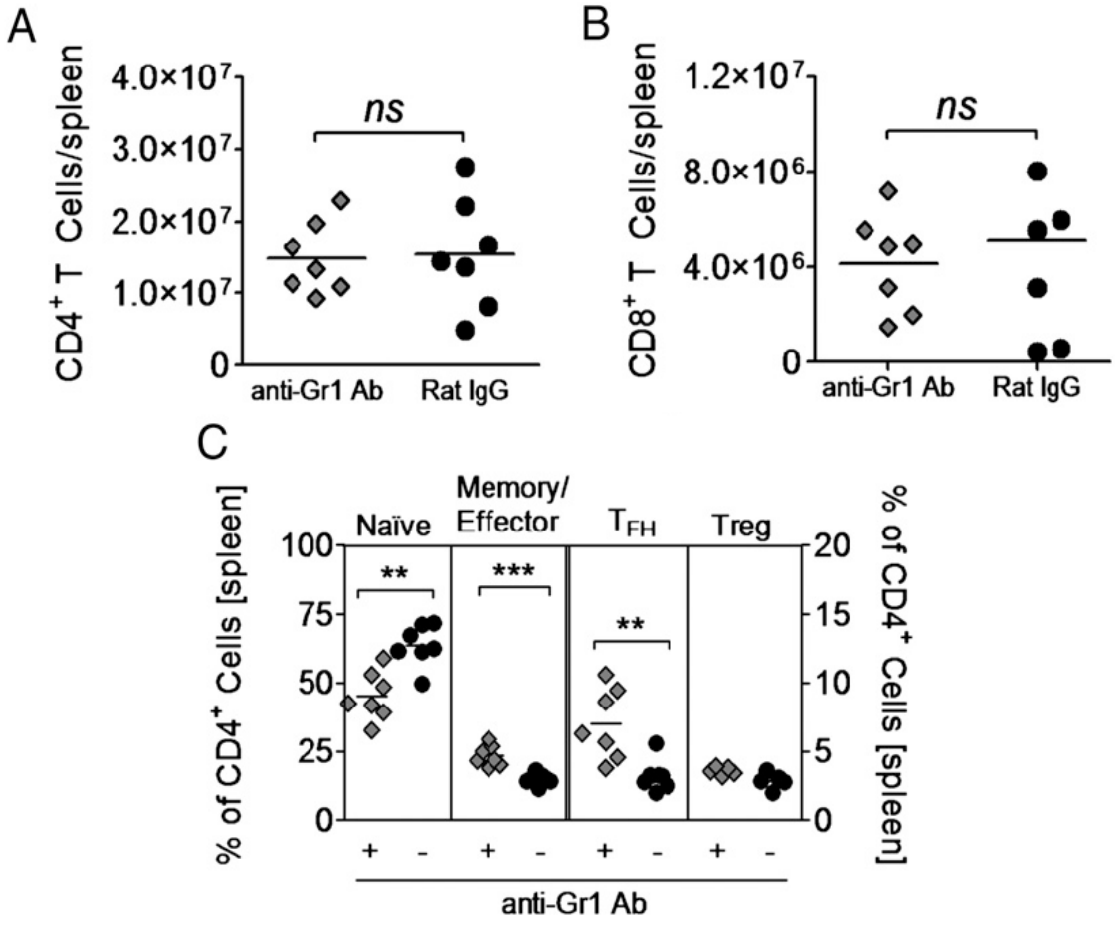

Figure 3.5 Depletion of Gr1$^+$ cells affects CD4$^+$ T cell subsets after antigen immunization in male (NZB X NZW)F1 mice. Male (NZB x NZW)F1 mice were immunized with NP_{27}-CGG in CFA on day 0 and treated with either anti-Gr1 depleting antibody or control rat IgG every three days starting at day -1. Mice were sacrificed 14 days post immunization and numbers of splenic CD4$^+$ T cells **(A)** and CD8$^+$ T cells **(B)** were determined. **(C)** Activation and differentiation of CD4$^+$ T cells were analyzed by flow cytometry: memory/effector CD4$^+$ T cells were identified as CD44^{high}CD62L^{low}CD4$^+$, T_{FH} cells as PD-1$^+$ICOS$^+$CD4$^+$, and Tregs as FoxP3$^+$CD4$^+$CD25$^+$. Each symbol represents one mouse. n = 6-7. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Mann-Whitney U test.

3.3.6 Gr1-expressing cells inhibit GC formation after Ag challenge

GC formation is crucial for class switching and production of Abs after immunization, and is dependent on T cell help, specifically T_{FH} cells. The increased number of GC B cells (Figure 3.2C) and T_{FH} cells (Figure 3.5C) in anti-Gr1 Ab–treated male (NZB x NZW)F1 mice 14 d post-immunization suggest that Gr1-expressing cells may also affect GC reactions. We tested this by comparing the number of T_{FH} cells and GC B cells, and the appearance of GCs in mice immunized with NP-CGG/CFA and treated with either depleting anti-Gr1 Ab or control rat IgG. Already at 5 d post-immunization, mice depleted of Gr1-expressing cells expressed elevated levels of T_{FH} cells, and at 7 and 14 d post-immunization, the levels were significantly higher in anti-Gr1 Ab–treated mice (Figure 3.6A). Numbers of splenic GC B cells were similarly elevated in mice depleted of Gr1-expressing cells 14 d post immunization ($p < 0.01$; Figure 3.6B). These data were further supported by immunofluorescence staining of spleen sections showing significantly elevated numbers and increased sizes of GCs in anti-Gr1 Ab–treated mice (Figure 3.6C, 3.6D).

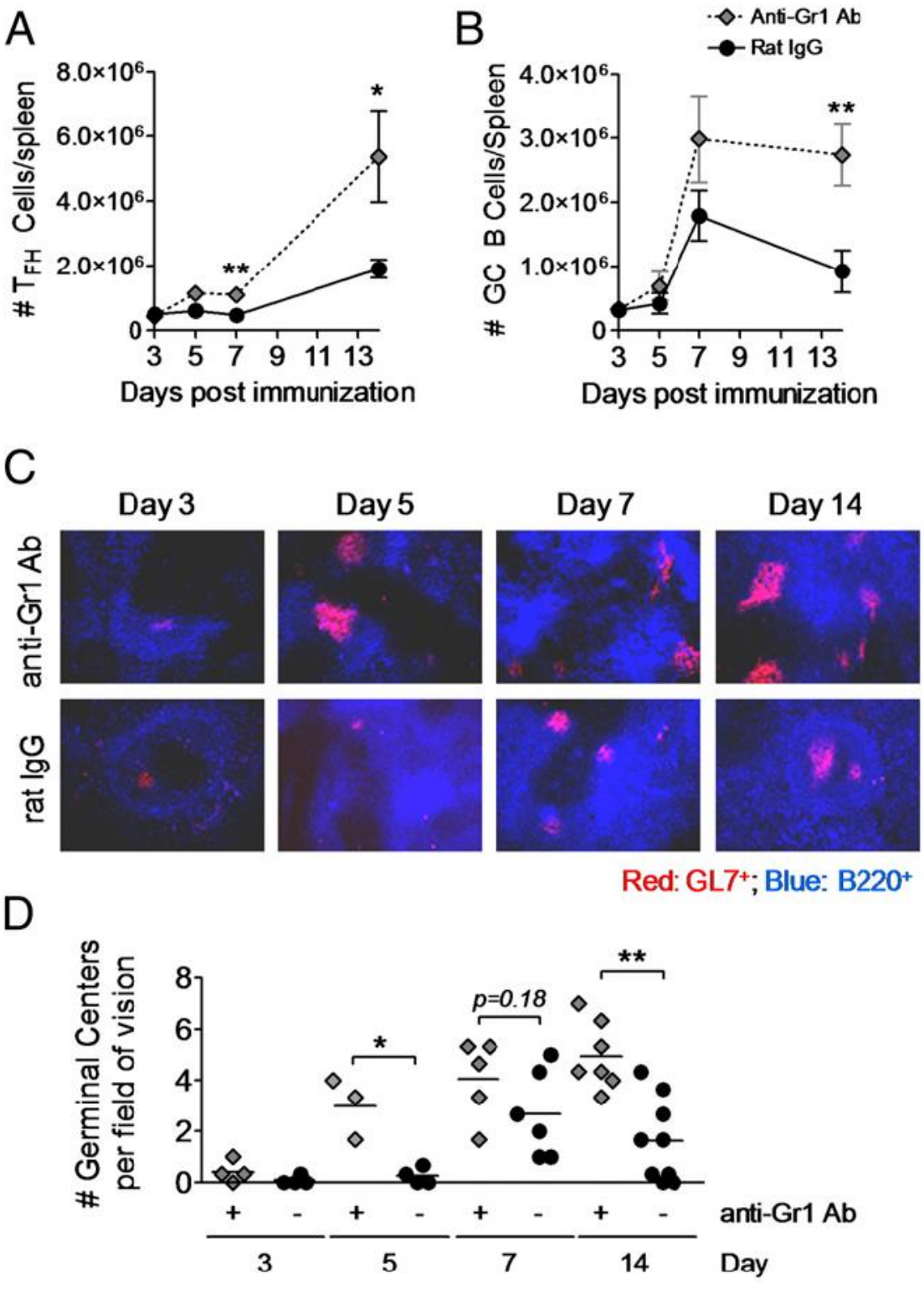
A
T_{FH} Cells/spleen
8.0×10⁶
6.0×10⁶
4.0×10⁶
2.0×10⁶
0
3 5 7 9 11 13
Days post immunization
**
*
B
Anti-Gr1 Ab
Rat IgG
GC B Cells/Spleen
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
3 5 7 9 11 13
Days post immunization
**
C
Day 3
Day 5
Day 7
Day 14
anti-Gr1 Ab
rat IgG
Red: GL7⁺; Blue: B220⁺
D
Germinal Centers per field of vision
8
6
4
2
0
*
p=0.18
**
+ - + - + - + -
anti-Gr1 Ab
3 5 7 14
Day

Figure 3.6 Depletion of $Gr1^+$ cells enhances T_{FH} cell differentiation and GC formation *in vivo*. (A and B) Male (NZB x NZW)F1 mice were immunized as described in Figure 3.1. Mice were additionally treated with anti-Gr1 depleting antibody or control rat IgG at days -1, 2, 4 and 6. Cohorts of mice were sacrificed at days 3, 5, 7, and 14 and analyzed for numbers of splenic T_{FH} cells ($CD4^+PD\text{-}1^+CXCR5^+$)**(A)** and GC B cells ($GL7^+B220^+$)**(B).** Numbers of TFH cells were compared per time point: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, Mann-Whitney *U* test. **(C)** Snap-frozen spleen sections from the same mice were stained for GC using biotinylated GL7/streptavidin-allophycocyanin (APC) and Pacific Blue-conjugated B220. Original magnification X10. **(D)** Numbers of GCs per field of vision were quantified by averaging the count from 3 distinct fields per spleen. n = 3-9 per time/point. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3.7 Male (NZB x NZW)F1 Gr1-expressing cells suppress T cell differentiation into T_{FH} cells *in vitro*

T_{FH} cells play a major role in the formation of GC. Because numbers of T_{FH} cells, GC B cells, GCs, and PCs were all significantly upregulated in the absence of Gr1-expressing cells, we asked whether $Gr1^+$ cells can directly suppress differentiation of naive T cells into T_{FH} cells *in vitro*. Sort-purified male (NZB x NZW)F1 naive T cells ($CD90.2^+CD62L^{high}$) were cocultured with increasing concentrations of sort-purified male $Gr1^+$ cells for 5 d in T_{FH} cell-skewing media (Figure 3.7A, 3.7B) (212). Upon harvest, cells were stained for Gr1, CD4, CXCR5, and PD-1, and the percentage of CXCR5, PD-1, double-positive $CD4^+$ cells was determined as previously described (212). $Gr1^+$ cells significantly suppressed the differentiation of naive T cells into T_{FH} cells. The suppression was dose dependent and reached statistical significance at 4:1 and 8:1 ratios of T cells to $Gr1^+$ cells ($p < 0.01$). The suppression of differentiation was not due to reduced survival of T cells, as the number of CD4+ T cells recovered on day 5 improved significantly and in a dose-dependent manner with the addition of Gr1+ cells ($p< 0.05–0.001$; data not shown). Thus, immunosuppressive $Gr1^+$ cells appear to play a hitherto unrecognized role in controlling naive T cell differentiation during T-dependent Ag exposure.

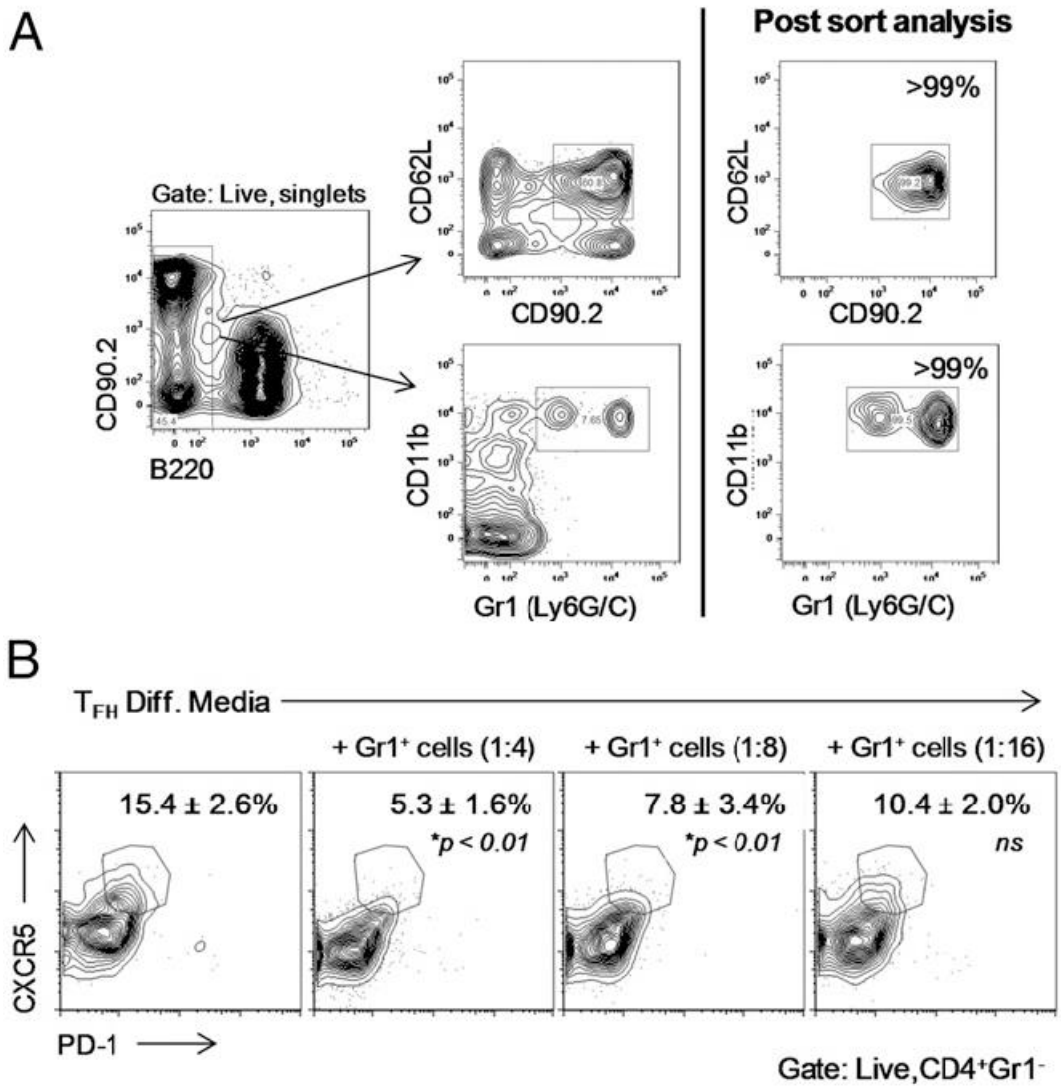

Figure 3.7 Male (NZB x NZW)F1 Gr1$^+$ cells inhibit naïve T cell differentiation into T_{FH} cells *in vitro*. **(A)** Naïve CD4$^+$ T cells (CD90$^+$CD62L^{high}) and total Gr1$^+$CD11b$^+$ cells were flow sorted from 9 wk old male (NZB x NZW)F1 mice by flow cytometry. **(B)** T cells were plated in T_{FH}-skewing media alone or in the presence of Gr1$^+$CD11b$^+$ cells at the indicated ratios. After 5 days, cells were harvested and stained for CD4, CXCR5 and PD-1, indicating a T_{FH}-like phenotype. All cells analyzed were Gr1$^-$. The contour plots shown are representative of four independent experiments. The mean ± SD per condition

is shown in the upper right corner of each plot. The *p* value given in each plot indicates the statistical difference between T cells differentiated in the presence or absence of $Gr1^+$ cells (one-way ANOVA test).

3.4 Discussion

The sex bias in SLE and other autoimmune diseases is not well understood. There is evidence that sex hormones, both estrogen and testosterone, are involved in the differential prevalence of SLE and mouse lupus-like disease among males and females (70,72,73,81,169,170). The protective function of testosterone, although well established, has not been studied in details and the molecular mechanism through which male sex hormone may act is not known. Interestingly, Ab responses to immunization are often also higher in females than in males (reviewed in Ref.(210)), suggesting that a generally overactive immune system in females might exist and help explain the female sex bias observed in many autoimmune disorders, including SLE.

We have recently shown that male lupus-prone (NZB x NZW) F1 mice have higher levels of immunosuppressive $Gr1^+$ cells than females, that these cells are driven by testosterone, and that depletion of the cells in males resulted in increased spontaneous autoantibody production (211). Using the same model, in this article, we describe how male (NZB x NZW)F1 mice respond less vigorously than females to TD exogenous Ag immunization. Furthermore, depletion of $Gr1^+$ cells in (NZB x NZW)F1 males increased the Ab response to Ag challenge, along with an underlying expansion of splenic GC B cells, PCs, and TFH cells. The main function of T_{FH} cells is to facilitate GC reactions and the differentiation of naïve B cells into PCs. Interestingly; T_{FH} cells have been associated

with disease severity in lupus patients, as well as several murine models (154,161,207,215-218). Thus, we hypothesized that $Gr1^+$ cells control Ab production primarily via modulation of T_{FH} cells. In support hereof, we found that $Gr1^+$ cells inhibited the differentiation of T_{FH} cells *in vitro* in a dose-dependent manner, although the mechanism of suppression remains unresolved.

Gr1+ cells are a heterogeneous population of cells consisting of mature and immature neutrophils, immature monocytes, and some dendritic cell subsets. Both proinflammatory and suppressor functions have been associated with such cells. In cancer, immunosuppressive Gr1+ cells are widely known as myeloid-derived suppressor cells (MDSCs; reviewed in Ref.(175)). Despite the identification of immunosuppressive Gr1+ cells in several models of autoimmunity (125,126,219-221), during viral infections (123), and graft rejection (222), the term MDSC is only sporadically used in such models. Whether testosterone-induced Gr1-expressing cells from male (NZB x NZW)F1 mice can be classified as MDSCs or represent a different population of immunosuppressive Gr1-expressing cells in this lupus model remains to be determined. The observation that male $Gr1^{high}CD11b^+$ cells suppress B cells independent of reactive oxygen species, NO, IL-10, and TGF-β (211) suggests the cells may not reflect tumor-induced MDSCs (121,223), although further molecular and functional studies directly comparing these cell subsets are needed to confirm such distinction.

In nonautoimmune C57BL/6 mice, Gr1-expressing cells have also been shown to affect the activation of adaptive immunity after footpad immunizations (224). Specifically, it was shown that neutrophils (defined as $Gr1^+Ly\text{-}6G^+CD11b^+$) interfered with the activation of T cells through the control of Ag uptake by professional APCs, thereby

limiting dendritic cell–T cell contact time in draining lymph nodes (224). Interestingly, analyses of $Gr1^+$ cells in nonautoimmune mouse strains revealed similarly elevated levels in males of most strains, including C57BL/6 (B6), BALB/c, and 129/Sv (data not shown). Upon immunization with NP-CGG in CFA, we detected increased serum Ab responses in female as compared with male B6 mice (data not shown). Interestingly, preliminary analyses showed that Ab-mediated depletion of $Gr1^+$ cells in B6 mice drove expansions of GC and memory B cells in spleens of both male and female C57BL/6 mice 4 wk after immunization, albeit without affecting anti-NP–specific Ab levels (E. Der and T.N. Jorgensen, unpublished observations). Thus, $Gr1^+$ cells from nonautoimmune B6 mice may exert some, but not all, of the immunosuppressive functions we observe in (NZB x NZW)F1 mice.

Interestingly, for reasons yet unknown, Gr1-expressing cells seem to have a limited protective effect in females (Ref. (211)) and this study). In fact, upon aging or during active disease, female $Gr1^+$ cells become stimulatory as described by us and others (113,211,225,226), suggesting that disease-related inflammatory factors present in lupus-prone females can influence the immunosuppressive function of immature myeloid $Gr1^+$ cells. Identification of such factor(s) clearly represents an attractive new target for therapeutic use in SLE and other diseases with dysregulated Ab production.

In summary, Gr1-expressing cells from lupus-prone male (NZB x NZW)F1 mice suppress T and B cell responses, resulting in reduced GC formation and less PC differentiation. Discovering the mechanism through which $Gr1^+CD11b^+$ cells suppress in the context of lupus could open new doors for immunotherapy of lupus patients and new targets for therapeutic agents.

Acknowledgements

We thank Jennifer Powers for invaluable help with all flow cytometry– based cell sorts and Dr. Eric Pearlman for purified RB6-8C5 anti-Gr1 Ab.

CHAPTER IV

INTRINSIC AUTOIMMUNE CAPACITIES OF HEMATOPOIETIC CELLS FROM FEMALE NEW ZEALAND HYBRID MICE

Abstract

Most systemic autoimmune diseases are more commonly found in females than in males. This is particularly evident in Sjögren's Syndrome, Systemic Lupus Erythromatosis (SLE) and thyroid autoimmunity, where the ratio of females to males ranges from 20:1 to 8:1. Our understanding of the etiology of SLE implies important roles for genetics, environmental factors as well as sex hormones, but the relative significance of each remains unknown. Using the New Zealand hybrid mouse model system of SLE we present here new fetal liver chimeras –based system in which we can segregate the effects of immune system genes and sex hormones *in* vivo. We show that female hematopoietic cells express an intrinsic capacity to drive lupus-like disease in both male and female recipient mice, suggesting that this capacity is hormone independent. Particularly, only chimera mice with a female hematopoietic system showed significantly increased numbers of germinal center B cells, memory B cells and plasma cells followed by a

spontaneous loss of tolerance to nuclear components and hence elevated serum anti-nuclear autoantibodies. A protective effect of testosterone was noted with regards to disease onset, not disease incidence. Thus, genetic factors encoded within the female hematopoietic system can effectively drive lupus-like disease even in male recipients.

4.1 Introduction

Autoimmune diseases such as systemic lupus erythematosus (SLE) have a strong female bias (64). A female predominance is also observed in the New Zealand hybrid mouse model of SLE ((NZB x NZW)F1), where 100% of females, but less than 40% of males develop end-stage renal disease within 1 year of age (70,71) . Lupus-like disease in (NZB x NZW)F1 mice is characterized by elevated anti-nuclear autoantibodies (ANA), IgG-immune complex (IgG-IC) deposition and complement fixation in the kidney glomeruli, and glomerulonephritis (GN) resembling the human disorder (227). The disease is generally believed to be mediated by immune system defects as shown in bone marrow (BM) transfer studies (228).

Levels of sex hormones or differences in sex-linked gene expression patterns are valid explanations for the pronounced sex difference observed (NZB x NZW)F1 lupus-like disease. In this regard, prepubertal hormonal manipulation studies have shown a protective effect of testosterone and exacerbating effect of estrogens (70,72,73,81,208). In addition, exposure to sex hormones during embryogenesis can affect autoimmune development in adult mice (229).

Genetic overexpression of X-linked genes, as seen in mice carrying the *Yaa* lupus susceptibility locus, has also been strongly associated with disease development (161,230). Particularly, a link between copies of *Tlr7* and the development of ANA have been demonstrated (161,231-233), although other genes expressed on the X chromosome likely also play a role, as demonstrated in TLR7-deficient male B6.Nba2(*Yaa*) congenic lupus-prone mice (234). Also supporting an effect of the X chromosome are data showing a correlation between pristine-induced lupus-like disease and X chromosome dosage in castrated Sry-transgenic male mice and the accelerated spontaneous development of lupus in XX versus XY^- NZM2328 mice (157).

Type I interferons (IFNα) play a crucial role in SLE and lupus-like disease development (187,235). In (NZB x NZW)F1 mice, elevating the levels of IFNα increases autoantibody production and accelerates renal disease onset (236). IFNα can be produced by many cell subsets, but most noticeably by plasmacytoid dendritic cells (pDCs) in response to a variety of stimuli targeting intracellular toll-like receptors (TLR) 7, 8 and 9 and cytoplasmic DNA sensors such as Aim2, DAI/ZBP1, Lrrfip1 and IFI16 (Ifi204) (237-240). IFNα is known to affect T cells, as well as B cells, although whether one or both mechanisms are involved in IFNα-driven lupus-like disease is still unknown (241,242).

In this study, we investigated whether female hematopoietic stem- and progenitor cells were capable of driving autoimmunity in the presence of male and/or female sex hormones. Using a unique mixed sex chimera system, we found that female hematopoietic cells (HCs) could drive the development of lupus-like renal disease, elevated levels of germinal center (GC) B cells, memory B cells and plasma cells, and increased ANA in all recipients regardless of sex hormone levels. In addition, mice

receiving female HCs expressed elevated levels of serum IFNα prior to the generation of ANA and the onset of renal disease. Male recipients of female HCs exhibited a delay in the onset of disease as compared with female recipients, suggesting that the protective effect of testosterone affected early events in disease propagation only. Thus, surprisingly the disease-driving capacity of female HCs appears more potent in driving disease than the known protective effect of testosterone.

4.2 Materials and Methods

4.2.1 Mice and cells

Three week old male and female (NZB x NZW)F1 mice were obtained from The Jackson Laboratory and kept in a specific pathogen-free environment at National Jewish Health (Denver, CO, USA). All mouse experiments were approved by the local IACUC committee. Bone Marrow chimera mice were generated by lethal irradiation (1000 rad) using a Cs^{137}-irradiator of 4 wk old male or female prepubertal (NZB x NZW)F1 mice. Mice were given acidified water (pH 2.7) to drink throughout the experiment. Bone marrow cells were obtained from 4 wk old male or female (NZB x NZW)F1 mice and $5x10^6$ cells were injected intravenously 2-4 hours after irradiation. Fetal liver single cells were obtained from E13.5-E14.5 embryos and frozen in 90% fetal bovine serum, 10% DMSO at -80°C. The sex of each embryo was determined visually as well as by real-time RT-PCR analyses of *Uty* and *Xist* expression levels (see below). Each fetal liver provided enough cells to reconstitute 3 recipients via tail vein injection.

4.2.2 Real-time reverse transcriptase-polymerase chain reaction

PBMCs were obtained from chimera mice 6 weeks post reconstitution. RNA was isolated using RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA) and cDNA prepared using the qScript cDNA SuperMix (Quanta BioSciences, Gaithersburg, MD, USA). PCR was run on a real-time PCR machine (ABI7300, Applied Biosystems, Carlsbad, CA, USA) using the following primers: *Xist*-F: 5'-GGAGGAACGAAAGACCAAATTG-3'; *Xist*-R: 5'-GTCCCACCCTCTGT GAGTGAA-3'; *Uty*-F: 5'-TGCCATCACAAGTCAAAGCAA-3'; *Uty*-R: 5'-TGGTGCATCCA ACCTAACTGTT-3'. Unmanipulated male and female samples were used separately or in different ratios (1:1, 1:3, 1:9, 1:27) to generate a standard curve. The levels of transcripts in chimera mice were calculated as % of total.

4.2.3 Detection of sex hormones

Estradiol and testosterone were measured by ELISA using the manufacturer's protocols (US Biological, Salem, MA, USA). Testosterone levels were measured on 10 times diluted serum. Estradiol levels were measured after extraction. Briefly, 50 µl of serum was vortexed with 500 µl ethyl ether for 30 seconds. Phases were allowed to separate and the organic phase was transferred to a fresh glass tube. Solvent was allowed to evaporate before the residue was dissolved in 250 µl of extraction buffer.

4.2.4 Antibody ELISA

Serum was obtained from chimera mice every 4 weeks starting 8 weeks post irradiation and reconstitution. For detection of total IgG and IgM, serum was diluted 1:50.000–1:200.000 in serum diluent (sterile filtered 0.5% bovine γ-globulin, 5% gelatin, 0.05mM

Tween in 1x PBS). For detection of ANAs, serum was diluted 1:300. Levels of anti-chromatin, anti-histone and anti-dsDNA IgG autoantibodies were measured as previously described (211). All reactions were developed using 10mg/ml 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)(ABTS) in McllWain's buffer (0.09 M Na_2HPO_4, 0.06 M citric acid, pH 4.6). Anti-dsDNA IgG levels were determined using the manufacturer's protocol (Alpha Diagnostic International Inc., San Antonio, TX, USA).

4.2.5 Cytokine ELISA

IFNα and BAFF were measured in 1:4 diluted serum obtained 12 and 16 weeks post irradiation and reconstitution, respectively, using the manufacturer's protocols (IFNα ELISA: PBL Interferon Source, NJ; BAFF ELISA: R & D Systems, Minneapolis, MN, USA).

4.2.6 Flow Cytometry

Flow cytometry was performed using a Cyan Flow cytometer ADP (BECKMAN COULTER, Indianapolis, IN, USA) and all analyses were done using FloJo version 9.5.2 (Tree Star Inc., Ashland, OR, USA). Antibodies with the following specificities were used for all analyses: CD11b, CD11c, CD19, CD21, CD23, CD38, CD40, B220 (CD45R), CD86, CD138, F4/80, Gr1 (Ly6C/6G), IgM, IgD, (all from eBiosciences, San Diego, CA, USA). Peanut agglutinin (PNA) was obtained from Vector inc. (Burlingame, CA, USA).

4.2.7 Immunofluorescence staining

IgG deposition and complement factor 3 (C3) fixation was measured by immunofluorescence staining. Briefly, half kidneys were quick-frozen in OCT™ and 5µm sections were prepared. Sections were stained using TexasRed-conjugated anti-mouse IgG (Invitrogen, Grand Island, NY, USA) and FITC-conjugated anti-mouse C3 specific antibodies (ICL Inc., Portland, OR, USA). Images were collected using an HC Plan Apo 20x/0.7NA objective lens on a Leica DMR upright microscope (Leica Microsytems, Buffalo Grove, IL, USA) equipped with a Retiga EXi Cooled CCD Camera (QImaging, Surrey, BC, Canada).

4.2.8 Statistical analysis

All statistical analyses were done using GraphPad Prism v. 5.04 (GraphPad INC., La Jolla, CA, USA). Analyses of cumulative incidence were done using a Log-rank test (Mantel Cox test). Comparisons of average time of onset, cellular proportions, and serum cytokine levels between two groups were done using a two-tailed non-parametric Mann Whitney test. *P* values < 0.05 were considered statistical.

4.3 Results

4.3.1 Female BM cells transfer renal disease into male and female recipients with a higher incidence and faster kinetic than male BM cells

Estrogens are known to promote lupus-like disease development in (NZB x NZW)F1 mice, while testosterone has been found to protect against the disease (72,73). In addition, X chromosome dosage has been found to affect disease development in other mouse models of SLE (157,243) . Since sex hormones affect the immune system

(76,244), distinguishing the effect of hormones from the effect of genes has been challenging. We asked if female HCs from (NZB x NZW)F1 mice could transfer disease into hormonally intact, lethally irradiated, age-matched male recipients. The major male antigen H-Y is not presented by H2d and H2z, allowing reconstitution to occur without rejection (245).To avoid potential effects of pubertal sex hormones, we performed the experiments using 4 wk old, prepubertal mice. Female HCs were capable of driving lupus-like renal disease in 100% of recipient mice by 31 wks post transfer, regardless of the sex of the recipient mouse (Figure 4.1A). In contrast, within the same timeframe, male HCs transferred disease into 57% and 25% of female and male recipients, respectively (Figure 4.1A, $p < 0.05$-0.001). Even when kept until 1 year of age (48 weeks post transfer), only ~85% of recipient mice accepting male hematopoietic cells developed renal disease ($p<0.001$). Irradiation and reconstitution itself accelerated end-stage lupus-like disease development in all recipient mice regardless of sex, although the characteristic difference between control male-into-male and female-into-female remained statistically significant ($p < 0.001$). The disease onset was similar in male and female mice receiving female BM cells, while M-into-M BM chimera mice started developing disease slightly later than M-into-F BM chimera mice (Figure 4.1A, not statistically significant).

The differential disease development was not driven by differences within the stem cell and progenitor cell compartment of males and females, as BM samples from 4 wk old unmanipulated male and female (NZB x NZW)F1 mice showed equivalent levels of hematopoietic stem cells (HSC), common myeloid progenitors (CMP), common lymphoid progenitors (CLP), granulocyte-macrophage progenitors (GMP) and

megakaryocyte-erythrocyte progenitors (MEP) (Figure 4.1B). In addition, all mice analyzed had grafted successfully, as noted by the relative expression of *Xist* and *Uty* transcripts in PBMC fractions from mice receiving female or male hematopoietic cells (Figure 4.1C). Moreover, recipient mice continued to express sex hormones at levels equivalent to unmanipulated mice as determined by serum levels of estradiol and testosterone (Figure 4.1D-E). Thus, female HCs from prepubertal 4 wk old (NZB x NZW)F1 mice transferred accelerated renal disease into both male and female age-matched (NZB x NZW)F1 mice independently of the recipient's sex hormone environment.

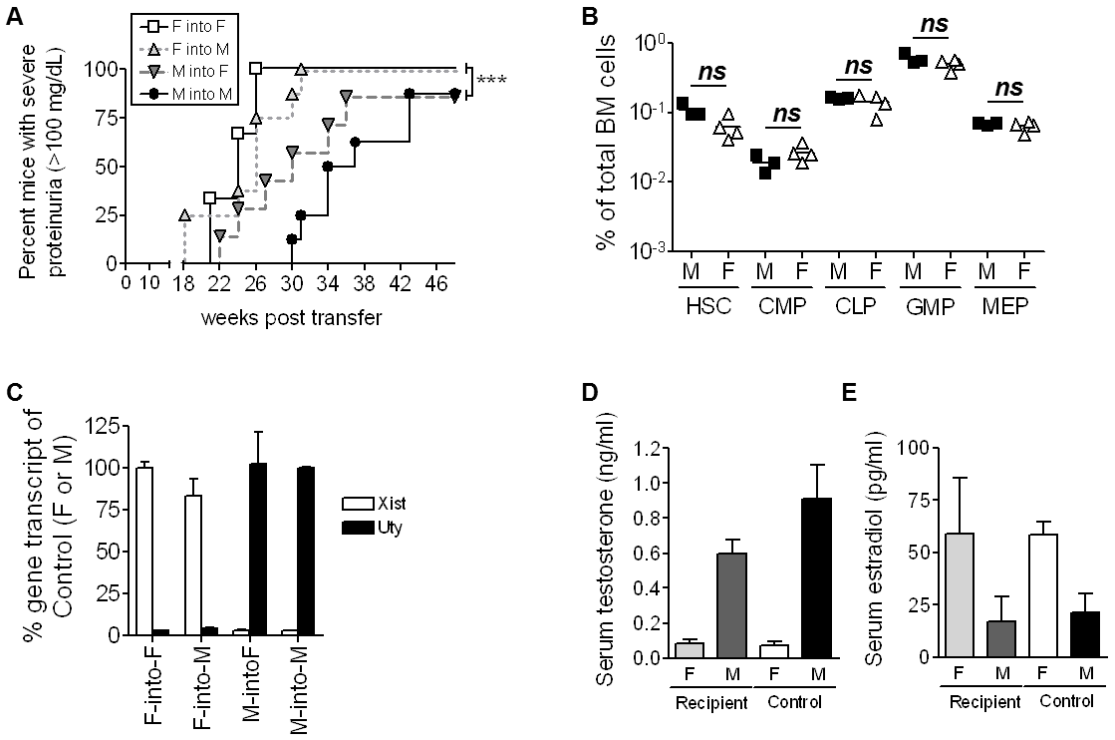

Figure 4.1 Female prepubertal BM cells transfer lupus-like disease in a hormone independent fashion. Four wk old (NZBxNZW)F1 male and female mice were lethally

irradiated and reconstituted with male or female BM cells from age-matched mice. **(A)** Mice were followed for the development of renal disease by detection of proteinuria every two weeks. Mice with severe proteinuria (> 100mg/dL on two consecutive readings) were considered positive. All mice were euthanized 48 weeks post transfer, regardless of disease stage. Female-into-female (open square, *n*=6); Female-into-Male (light grey triangle, *n*=8); Male-into-female (dark grey triangle, *n*=7); Male-into-Male (filled circle, *n*=8). **(B)** BM cells were isolated from 4 wk old mice (*n*=5 for both males and females) and the proportions of hematopoietic stem cells and progenitor cell subsets were determined by flow cytometry. **(C)** PBMCs were isolated from BM chimera mice 18 weeks after transfer (*n*=2 of each). Total RNA was isolated and cDNA generated. The levels of Xist and Uty transcripts were normalized to the levels of beta-2-microglobulin and the % was calculated relative to the levels in control female-into-female (100% Xist) or male-into-male (100% Uty) BM chimera mice. **(D and E)** Serum was isolated from BM chimera mice 18 weeks after transfer and levels of testosterone **(D)** and estradiol **(E)** were measured by ELISA. Female recipient: n=13 (testosterone), n=6 (estradiol); male recipient: n=15 (testosterone), n=5 (estradiol); female control: n=10 (testosterone), n=10 (estradiol); male control: n=6 (testosterone), n¼7 (estradiol). *** $p < 0.001$.

4.3.2 The capacity of female hematopoietic cells to transfer renal disease is present *in utero*

Sex hormones are produced at high levels starting at puberty. However, even in utero and during the postnatal period sex hormones are produced, and hence HCs from 4-week-old female (NZBxNZW)F1 mice could have acquired their autoimmune capacities as a result of such exposure. To test for this possibility, we generated fetal liver (FL) mixed chimera mice. FL cells were isolated from male or female (NZBxNZW)F1 embryos at days E13.5–E14.5 and transferred into lethally irradiated 4-week-old prepubertal male or female (NZBxNZW)F1 mice. Mice were followed for the development of proteinuria until 32 weeks after transfer. Diagnosis of disease was confirmed by discovery of elevated colocalized IgG-immune complex deposition and complement fixation in kidney glomeruli in chimera mice that had received female FL cells (Figure 4.2D).

Similar to the experiments involving BM cell transfer from 4-week-old donors, female FL cells induced a rapid onset of disease in 100% of recipient mice, while male FL cells induced less disease and significantly delayed disease onset (Figures 4.2A and B, $p<0.001$). However, disease occurred somewhat later in male, versus female, recipients of female FL cells (Figure 4.2B, $p<0.01$). Again, we did not find this to be a result of differences among the transferred HCs, as analyses of FL cells from male and female (NZBxNZW)F1 embryos showed no differences in the distribution of cell subsets (Figure 4.2C). Similar to the BM chimeric mice, serum level of sex hormones in FL chimeric mice were comparable to that of unmanipulated male and female (NZBxNZW)F1 mice (data not shown).

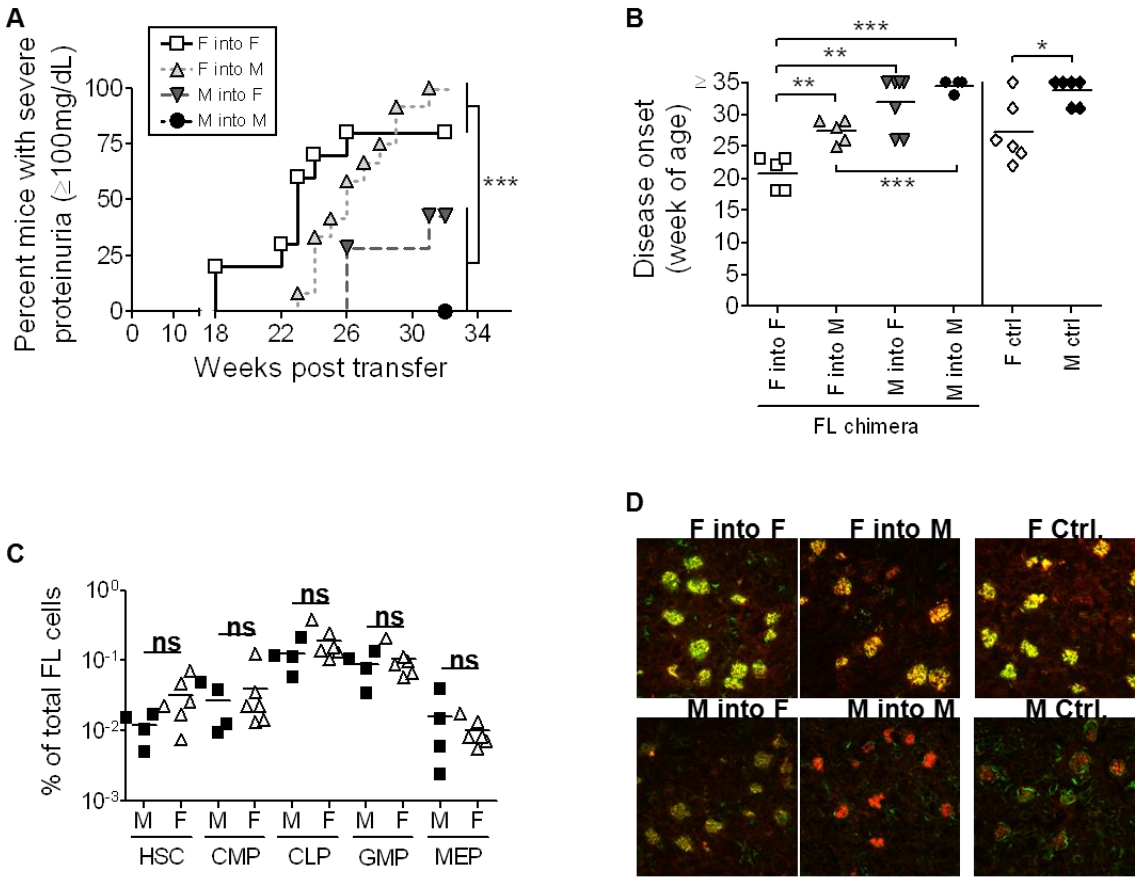

Figure 4.2 Female FL cells transfer lupus-like disease into both male and female recipients with 100% incidence. Four week old BWF1 male and female mice were lethally irradiated and reconstituted with male or female FL cells from E14.5 male or female (NZBxNZW)F1 embryos. **(A)** Two cohorts of FL chimera mice were followed for the development of renal disease by detection of proteinuria every 2 weeks. Mice with severe proteinuria (> 100mg/dL on two consecutive readings) were considered positive. All mice were euthanized 35 (cohort 1) or 32 (cohort 2) weeks after transfer, regardless of disease stage. Female-into-female (open square, *n* = 10); female-into-male (light grey triangle, *n* = 12); Male-into-female (dark grey triangle, *n* = 7); Male-into-male (filled circle, *n* = 4). **(B)** Disease onset up to 35 weeks after transfer (cohort 1) is shown.

Female-into-female (open square, n=5); female-into-male (light gray triangle, n=5); male-into-female (dark gray triangle, n=7); male-into-male (filled circle, n=4). Control unmanipulated mice are included for comparison: females (open diamonds, n=6) and males (filled diamonds, n=6). **(C)** FL cells were isolated from E14.5 embryos and the ratios of stem cell and progenitor cell subsets were determined in male cells (filled squares, *n*=4) and female cells (open triangles, *n*=6). NS: not statistically different. **(D)** Upon sacrifice of the mice described in **(A)**, kidneys were harvested and analyzed for IgG deposition (red) and C3 fixation (green). Pictures shown represent averages per condition. Each symbol represents an individual mouse. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.3.3 Reconstitution with Female FL cells specifically affects levels of post-activation B cell subsets

Lupus is a B cell and autoantibody mediated disorder. We tested if B cell numbers and subset distribution were different between the four groups of FL chimera mice. Gating strategies are depicted in Figures 4.3A-D. In spleens, neither total numbers of B cells ($CD19^{+}$) nor marginal zone B cells ($CD19^{+}CD21^{high}CD23^{-}IgM^{high}IgD^{low}$) were significantly different between various FL chimera mice (Figure 4.3 E and G). However, mice that had received female FL cells displayed overall increased levels of follicular mature B cells ($CD19^{+}CD21^{low}CD23^{high}IgM^{low}IgD^{high}$) (Figure 4.3F), regardless of the sex of the recipient. Even more strikingly, numbers of germinal center B cells ($CD19^{+}PNA^{+}CD38^{low}IgM^{low}$), memory B cells ($CD19^{+}CD38^{hi}IgM^{low}$) and plasma cells ($B220^{low/neg}CD138^{+}IgM^{-}$) were significantly elevated in chimera mice that had received female FL cells (Figure 4.3H-J, $p < 0.05$-0.01). Consistent with a female-driven effect

driving differentiation of mature B cells in the periphery, we found no differences among the relative levels of pro-B, pre-B and immature B cell subsets in the bone marrow of FL chimera mice (data not shown).

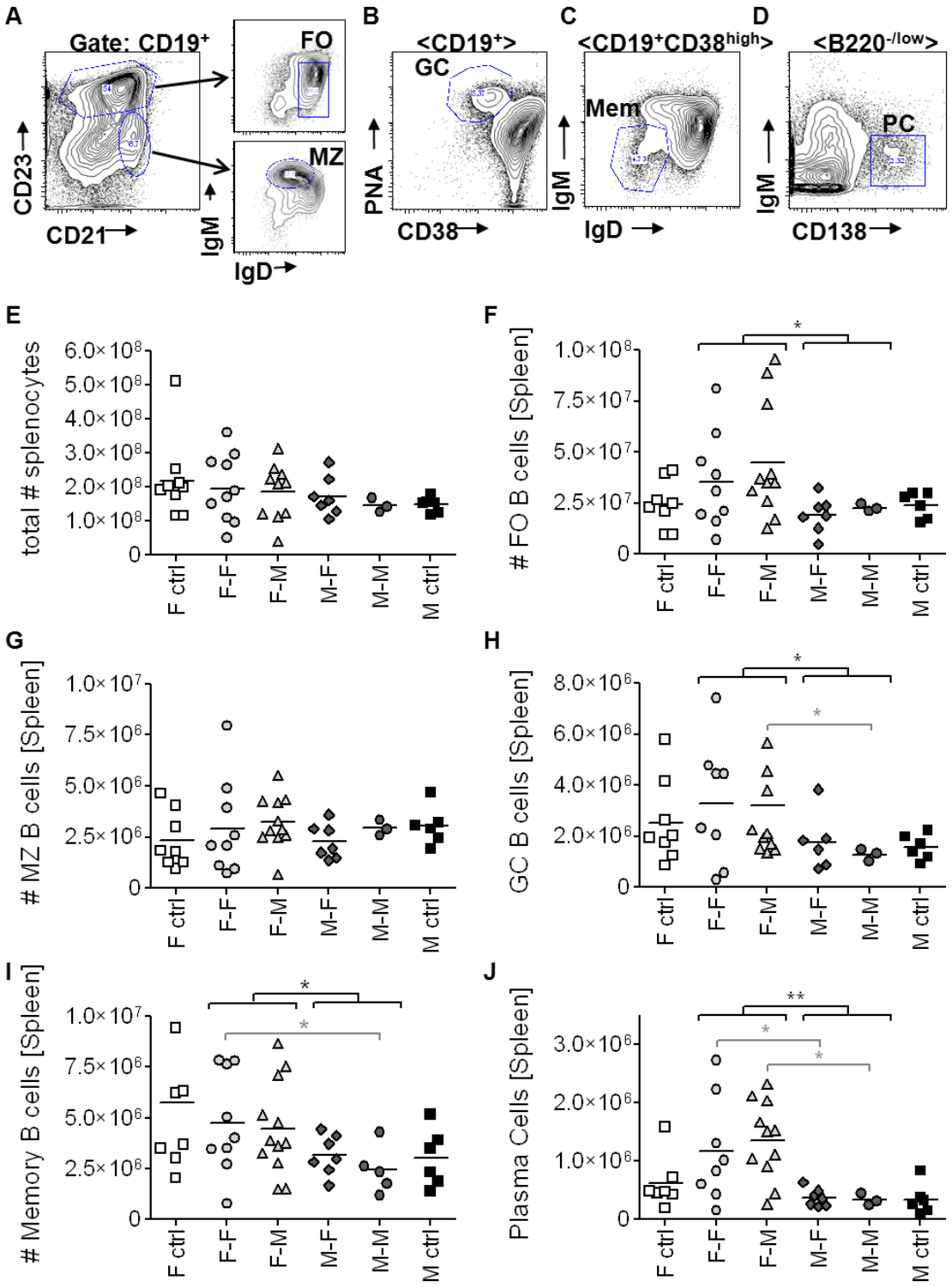

Figure 4.3 The female hematopoietic system of (NZB x NZW)F1 mice promotes B cell differentiation. Spleens were harvested from FL chimera mice at the time of

sacrifice. Samples were analyzed for the presence of B cell subsets by flow cytometry. **(A-D)** Representative plots show the gating strategy used to determine follicular mature (FO), Marginal Zone (MZ), germinal center (GC), Memory (Mem) B cells and plasma cells (PC). **(E-J)** Total numbers of indicated B cell subsets are presented per FL chimera mouse. Each symbol represents one mouse. * $p < 0.05$; ** $p < 0.01$. Brackets in black indicate statistical differences between groups receiving male or female FL cells, while brackets in grey represent statistical differences between individual groups of chimeras. Mice analyzed included female-into-female (n=9, female-into-male (n=11), male-into-female (n=7) and male-into-male (n=3) FL chimeric mice.

4.3.4 Autoantibody production is driven by female HCs and not affected by the presence of male sex hormone

Female (NZB x NZW)F1 mice develop hypergammaglobulinemia at early ages followed by a specific loss of tolerance to nuclear autoantigens (199,200). We analyzed whether total immunoglobulin and ANA levels were associated with the sex of the FL donor in our chimera system. Increased levels of total IgG, IgG_1 and IgG_{2A} in the serum of FL chimera mice did not correlate with the groups of mice developing renal disease (Fig. 4A-C). In fact, male-into-male FL chimera mice, which did not develop lupus-like disease, had the highest levels of circulating total immunoglobulins. In contrast, the levels of anti-chromatin IgG, anti-histone IgG and anti-dsDNA IgG were all significantly elevated in chimera mice that had received female FL cells as compared with those mice receiving male FL cells (Figure 4.4D-F, $p < 0.05$-0.001), suggesting that mice that had received female HCs displayed a specific loss of tolerance to nuclear antigens.

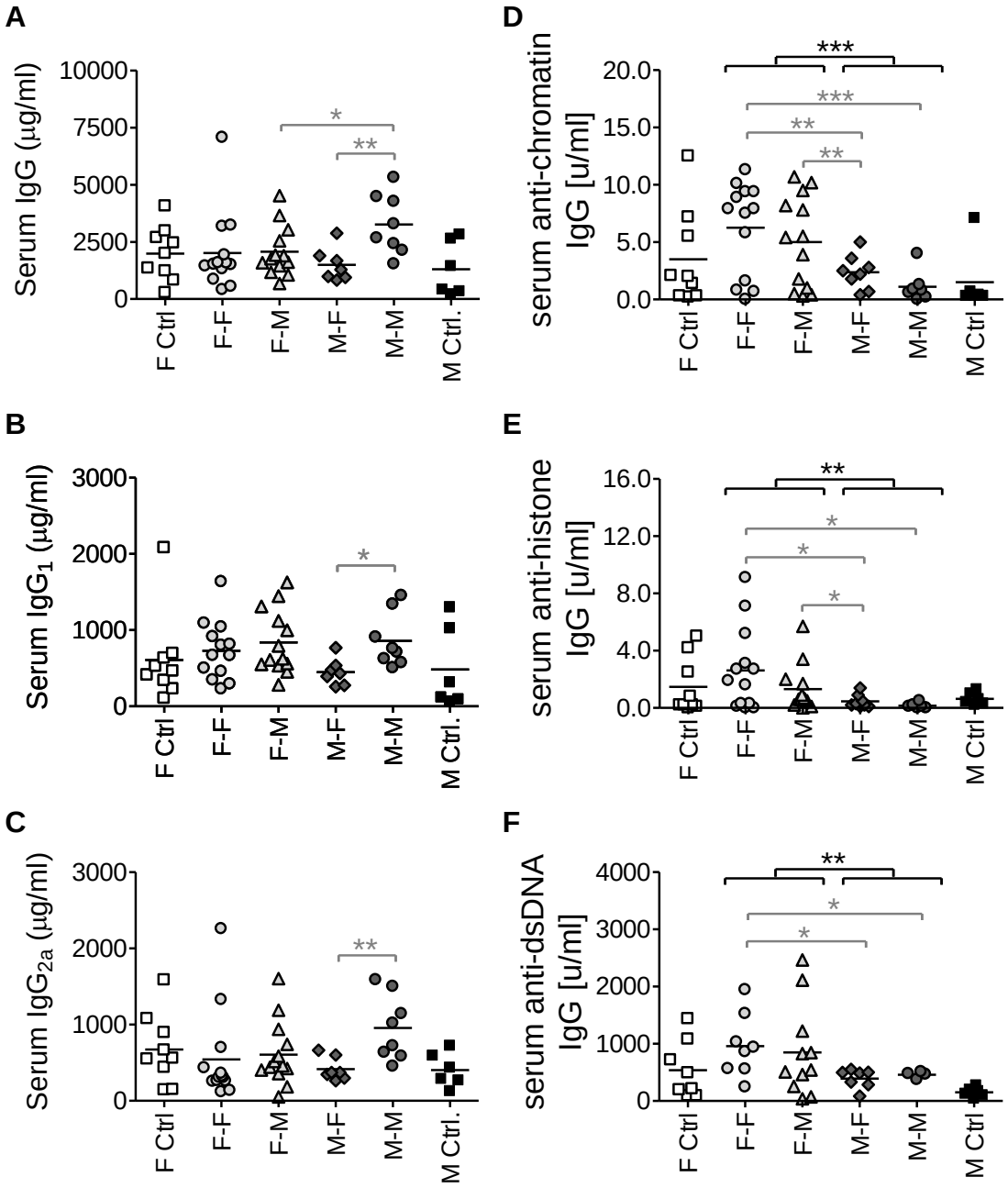

Figure 4.4 Serum anti-nuclear IgG autoantibody levels in FL chimera (NZB x NZW)F1 mice are elevated in mice that received female donor cells. Serum total IgG **(A)**, IgG1 **(B)**, IgG2a **(C)**, anti-chromatin IgG **(D)**, anti-histone IgG **(E)** and anti-dsDNA IgG **(F)** levels were measured by ELISA in samples from FL chimera mice at 12 **(A-C)** or 20 **(D-F)** weeks post irradiation/reconstitution. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Brackets in black indicate statistical differences between groups receiving male or female FL cells, while brackets in grey represent statistical differences between individual groups of chimeras. Groups of mice analyzed included female-into-female (*n*=8–13), female-into-male (*n*=11–13), male-into-female (*n*=7–8) and male-into-male (*n*=4–8) FL chimeric mice along with 6–9 female and 6 male unmanipulated age-matched controls.

4.3.5 Levels of serum IFNα, but not BAFF, are elevated in chimera mice receiving female FL cells

Possibly explanations for this observation include an increased capacity of female-derived B cells to differentiate into autoantibody producing cells or increased levels of B cell differentiating signals secreted by female-derived non-B cell HCs. To test the later idea, we examined FL chimera mice for levels of serum B-cell-activating factor (BAFF) at 16 weeks after irradiation and reconstitution; before the onset of renal disease. B-cell-activating factor (BAFF) is known to be involved in B cell survival and differentiation and has previously been found to be associated with lupus in several mouse models (14-16,246,247). FL chimera mice that had received female HCs did not express elevated levels of BAFF (Figure 4.5A). In fact, male FL chimera mice were found to express higher levels of BAFF than female FL chimera mice, regardless of whether these had received male or female FL cells ($p < 0.05$).IFNα is also known to influence B cell differentiation (248) and is capable of driving disease development in (NZB x NZW)F1 mice and related strains (97,236,249,250). We tested serum levels of IFNα in FL chimera mice 12 weeks post irradiation and reconstitution, before the onset of renal disease. Mice reconstituted with female FL cells displayed higher levels of serum IFNα than mice reconstituted with male FL cells (Figure 4.5B, female versus male donor: $p <$

0.05). Furthermore, levels of serum IFNα at 12 weeks after transfer correlated statistically with levels of serum anti-chromatin IgG ($p < 0.001$), anti-histones IgG ($p < 0.05$) and anti-dsDNA IgG ($p < 0.05$) measured 20 weeks after transfer (Figure 4.5C-E). Serum IFNα levels measured 12 weeks after reconstitution also trended towards a negative correlation with the onset of renal disease in all FL chimeras ($p = 0.1$, Figure 4.5F).

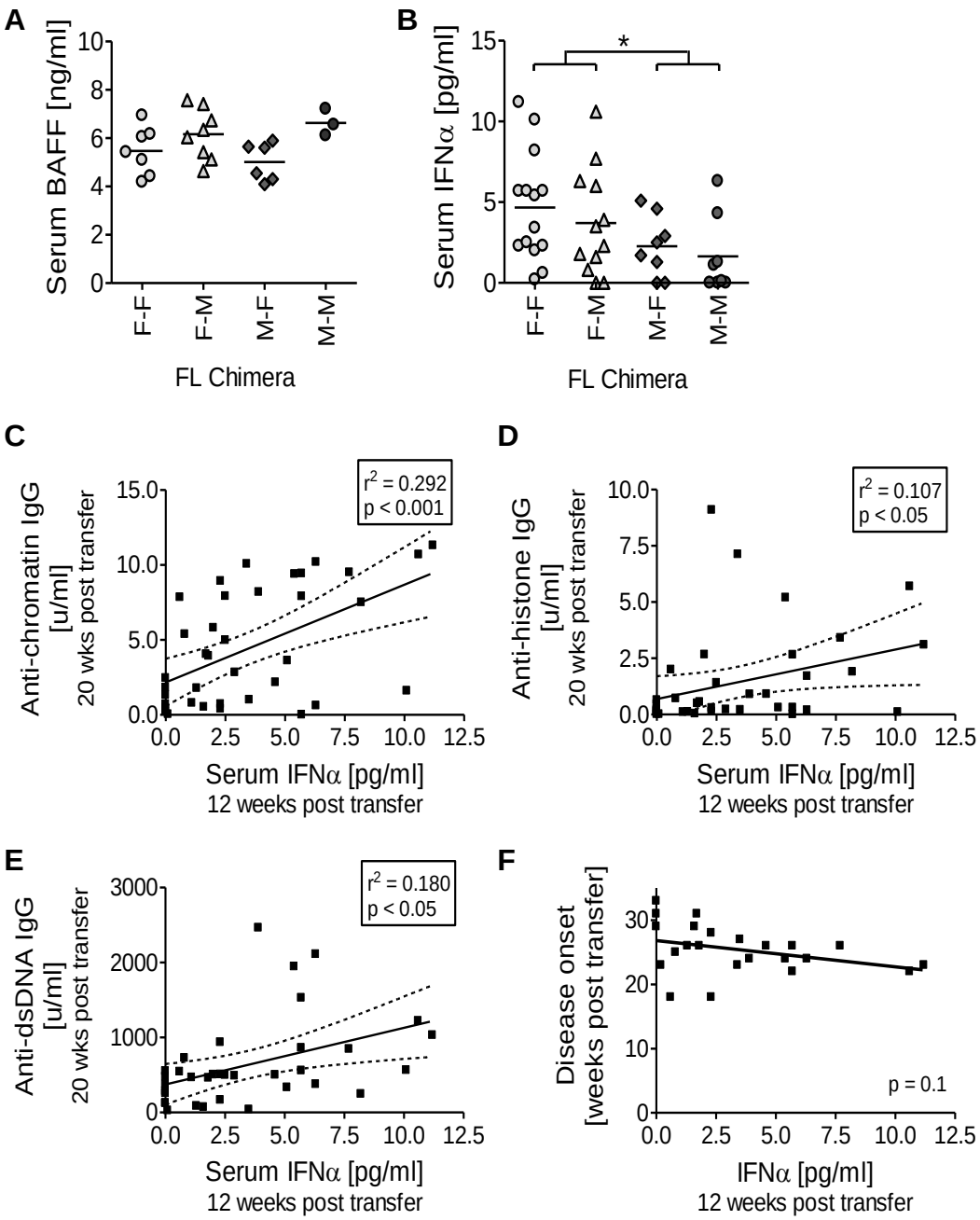

Figure 4.5 Elevated serum IFNα levels are driven by the female hematopoietic system and correlates with early disease onset. Serum was obtained from FL chimera mice 12 or 16 week after irradiation and reconstitution. Levels of BAFF were measured

16 weeks post transfer **(A)**, while levels of IFNα were measured 12 weeks post transfer **(B)**. Levels of IFNα correlated positively with autoantibody levels at 20 weeks after irradiation/reconstitution **(C-E)** and negatively with onset of disease **(F)**. Disease onset was defined as the time point where any given mouse presented with severe proteinuria (≥100 mg/dL) for the first of two consecutive readings (also see Figure 4.2). Each symbol represents one FL chimera mouse. * $p < 0.05$. Groups of mice analyzed included female-into-female (n= 7-14), female-into-male (n= 8-12), male-into-female (n= 6-8) and male-into-male (n= 3-8) FL chimeric mice.

4.4 Discussion

Although tremendous amounts of research have gone into determining the etiology of SLE, the underlying mechanism(s) driving disease initiation and/or progression are still poorly defined. We and others have previously shown that manipulation of sex hormone production from puberty significantly alters the development of renal disease (70,72,73,81,208). Specifically, castration of male lupus-prone (NZB x NZW)F1 mice was found to remove the protective effect of testosterone resulting in disease development equivalent to that of female unmanipulated mice (70,73). Conversely, ovariectomy of female (NZB x NZW)F1 mice prior to puberty fails to alter disease kinetics (73,81), suggesting that after the immune system is established in female mice around 2-3 weeks of age, estrogens are not crucial for disease progression. Here we have shown that, female HCs are capable of driving lupus-like disease in hormonally intact, lethally irradiated male (NZB x NZW)F1 FL recipient mice. Thus, even in the presence of testosterone, female HCs from lupus-prone (NZB x

NZW)F1 mice cannot be held back and proceed to generate autoreactive B cells, followed by IgG-IC deposition, glomerulonephritis and renal failure.

Pre-B cell lines from fetal livers of lupus-prone (NZB x NZW)F1 mice have previously been shown to possess intrinsic autoimmune competencies when compared with pre-B cell lines established from non-lupus prone strains (165), however, whether these cells lines were of a male or female origin was not reported and remains unknown. As the immune system of FL chimera mice originates from transferred stem cells and progenitor cells, rather than more mature lymphocytes, our data suggest that the defect is genetically encoded. A major player in B cell development and differentiation is Bruton's tyrosine kinase (Btk) encoded by the X chromosome (251). Although not much is known about Btk levels and activity in lupus, a recent study reported amelioration of end-stage lupus-like disease in older female (NZB x NZW)F1 mice treated with Btk inhibitor (252). The study did not investigate if males were equally susceptible, and thus any sex-driven abnormality remains to be identified. Another candidate gene is Cd40L, also encoded by the X chromosome. CD40L is essential for T-cell-dependent B-cell activation and has been assigned an essential role in (NZBxNZW)F1 lupus-like disease development;(11,253) however, whether CD40L-mediated B-cell activation is differentially active in male and female recipients remains unknown.

IFNα is recognized as a key cytokine in SLE and mouse lupus-like disease (97,101,235,249,250,254). IFNα is predominantly induced in response to viral or intracellular bacterial infections, but although tempting, a cause-and-effect relationship between infections and SLE remains elusive. We observed elevated levels of serum IFNα

in chimera mice that had received female HCs, suggesting that dysregulated IFNα production, either directly or via exorbitant endogenous stimuli, is also intrinsically driven by female HCs. Several genes known to be involved in IFNα production and/or responsiveness have been associated with SLE in genome wide association studies, including *Irf5*, *Irf7*, *Irf8*, *Irak1*, *Tyk2*, *Stat4* and *Fcgr2a* among others (reviewed in (255)). Of these, *Irak1* is particularly interesting. First, the *Irak1* gene is encoded on the X chromosome making it an attractive candidate when studying sex-dependent disease patterns. Secondly, IRAK1 protein is essential for most TLR-mediated intracellular signaling resulting in IFNα production. And finally, IRAK1 has been shown to be required for SLE serum (i.e. IgG-IC)-induced pDC activation and IFNα production (256).

Tlr7 is another X-linked gene that has been strongly associated with mouse lupus-like disease (234,243), although not directly identified in GWAS studies. Recently, however, TLR7 expression levels were found to correlate with elevated levels of anti-RNA antibodies in SLE patients (257). TLR7 is expressed by both pDCs and B cells resulting in IFNα production and B cell differentiation (94,258), and TLR7-deficient lupus-prone mice fail to develop significant levels of ANAs (243). Whether female pDCs and/or B cells respond better to TLR7 cross-linking than male cells remains to be determined, however, it is tempting to speculate such a relationship since (1) autoantibody producing $CD11c^{+}$ B cells depend on TLR7 signaling and are found predominantly in young autoimmune *females* (233), and (2) TLR7 agonist stimulation of human PBMCs resulted in significantly more IFNα production from female, than male cells (259,260).

As estrogen receptor expression is required for disease development in female (NZB x NZW)F1 mice, it is interesting to note that IFNα induces transcription of the Esr1 gene and subsequent expression of the estrogen receptor a (261,262). Oppositely, estrogen treatment has been suggested to drive dendritic cell activation and enhanced IFNα production upon TLR ligation (263,264). Whether male and female HCs respond equally to estrogens has been evaluated both *in vivo* and *in vitro* in (NZBxNZW)F1 and non-autoimmune mice, showing no significant differences (264-266). On the basis of these observations, we do not believe that disease–promoting effect of female HCs is because of increased responsiveness of female cells to low levels of estrogen present in male recipients, although further experiments are needed to firmly rule out this possibility.

So what about testosterone and its well established protective effect? We have recently described the presence of a population of testosterone-induced immunosuppressive myeloid cells ($Gr1^{high}CD11b^{+}$) in male (NZB x NZW)F1 mice (211). These cells have the capacity to directly suppress B cell differentiation *in vitro*, while depletion *in vivo* promotes autoantibody production in male mice. In our chimera system, male recipients experienced a later onset of disease, although autoantibody production seemed to be only marginally lower in male versus female recipients. On the basis of these observations, we propose that testosterone may act by promoting the development of immunosuppressive $Gr1^{high}CD11b^{+}$ cells capable of delaying, but not inhibiting, disease development. The presence of these cells is subsequently enough to control intrinsic disease promoting signals in *male* HCs, but not in *female* HCs, the latter promoting elevated IFNα production, B cell differentiation, ANA production and fatal renal disease. Taken together, we have found that (NZB x NZW)F1 female HCs have an

intrinsic ability to drive autoimmune lupus-like disease, regardless of the hormonal environment of the host. This strongly implicates genetic rather than hormonal factors as the underlying mechanism driving the increased incidence of autoimmunity in females as compared to males.

Acknowledgement

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Chapter V

SUMMARY AND DISCUSSION

1.5.1 Summary of major findings:

The studies in this work are concentrated on delineating the underlying causes for the strong female sex-bias seen in the (NZBxNZW)F1 mouse model of lupus-like disease. We have laid emphasis on the effect of sex hormones as well as the genetic factors that may be responsible for this sex-bias. We have investigated the cellular differences in the composition of male versus female immune system and have identified key cellular components playing a critical role in the progression of lupus-like disease in these mice. Also the relative role of sex hormone versus the intrinsic quality of the hematopoietic cells is examined using a unique BM chimera system. The major findings are as follows:

1. Male (NZBxNZW)F1 lupus prone mice harbor higher numbers of immune-suppressive $Gr1^{high}CD11b^{+}$ cells that suppress B cell differentiation *in vitro*.
2. Antibody mediated depletion of Gr1+ cells only in male (NZBxNZW)F1 leads to higher levels of auto-antibodies *in vivo*.

3. Female $Gr1^{high}CD11b^{+}$ cells lose suppressor in post-pubertal age and acquire a stimulatory function as the mice get old.
4. Male and female $Gr1^{high}CD11b^{+}$ cells use different mechanisms to suppress B cell differentiation.
5. Antibody mediated depletion of Gr1+ cells only in male (NZBxNZW)F1 leads to stronger T cell dependent antibody response.
6. $Gr1^{low}CD11b^{+}$ cells suppress T cell proliferation *in vitro*.
7. Depletion of Gr1+ cells leads to more T_{FH} cells and germinal centers.
8. Gr1+ cells suppress T_{FH} cells differentiation *in vitro.*
9. Hematopoietic cells from female (NZBxNZW) F1 mice can transfer renal disease to male recipients with higher incidence and faster kinetics.
10. Hematopoietic cells from female (NZBxNZW) F1have disease driving capacity *in utero.*
11. Auto-antibody production is controlled by the female hematopoietic cells and not the host sex hormone levels.
12. BAFF and IFN-α are elevated in recipients of female hematopoietic cells.

To summarize, there are multiple factors driving the lupus-like disease in (NZBxNZW)F1 mice and we cannot point out a single factor responsible for the sex-bias observed in the disease. The presence of testosterone driven immune-suppressive population of $Gr1^{high}CD11b^{+}$ and $Gr1^{low}CD11b^{+}$ in lupus-prone male (NZBxNZW)F1 with specific suppressor function is a novel finding. The elucidation of suppressive mechanism of male $Gr1^{+}CD11b^{+}$ can lead to novel therapeutic targets. Also the dissection of the testosterone dependent generation of $Gr1^{+}CD11b^{+}$ cells can lead to the

prospect of enhancing and maintaining these immuo-suppressive cells in female (NZBxNZW)F1 mice and modulate the progression of lupus-like disease. Testosterone driven immune-suppressive cells protect from the disease but in the BM chimera system the female hematopoietic cells take over the protective role of testosterone.

1.5.2 Discussion and future directions:

Sex-bias in autoimmunity is a well-studied and well documented phenomena. Our studies here try to address the relative contribution of major factors involved in the sex-bias we see in the (NZBxNZW)F1 mouse model of lupus-like disease such as immune cell composition, sex-hormones and genetic constitution of the immune system. As the (NZBxNZW)F1 is a multigenetic and spontaneous mouse model of lupus with a strong female bias, our findings possibly mimic the actual disease as closely as possible in an experimental system.

We have recently witnessed the emergence of various regulatory immune cell subsets like T_{regs}, B_{regs} and MDSCs. These regulatory cell types are commonly associated with immune-suppression which makes them prime targets for therapeutic interventions in diseases exacerbated by immune dysregulaiton such as autoimmunity and cancer. The role of T_{regs} and B_{regs} has been studied in autoimmune diseases but there are no definitive studies about their role in lupus. Also the role of MSDCs had been explored in other autoimmune conditions like EAE and type I diabetes but not in lupus. It is notable is that the effects of all these regulatory cell types have been studied only in the context of T cell immunity.

Our studies in Chapter 2, report a novel immuno-suppressive $Gr1^{high}CD11b^{+}$ (resembling granulocytic MDSCs) and $Gr1^{low}$ $CD11b^{+}$ (resembling monocytic MDSCs) MDSC like subsets in lupus prone (NZBxNZW) F1 mice. Interestingly, these cells are present in higher numbers in male (NZBxNZW) F1mice as compared to female (NZBxNZW) F1mice. It is to be noted that we have shown that the number of MDSC like cells are regulated by testosterone in (NZBxNZW) F1 mice; but it is not clear that the functionality of MDSC like cells described here are regulated by testosterone or not. It has been shown in studies that myeloid progenitors express androgen receptor but we have not been able to detect androgen receptor expression on myeloid progenitors in the (NZBxNZW) F1 mouse model. We speculate that BM stromal cells may be responsible for regulating the number of MDSC like cells in response to testosterone. *Further studies are needed to elucidate the exact mechanism of testosterone mediated regulation of* $Gr1^{+}$ $CD11b^{+}$. *Additionally, it needs to be addressed that if testosterone affects the* $Gr1^{+}$ $CD11b^{+}$ *cells directly or the effect of testosterone is mediated through a myelopoiesis promoting cytokine.*

We show that $Gr1^{high}CD11b^{+}$ cells suppress cytokine mediated B cell differentiation *in vitro* and that *in vivo* depletion of $Gr1^{+}CD11b^{+}$ lead to elevated production of auto-antibodies and kidney disease in male (NZBxNZW) F1. $Gr1^{low}$ $CD11b^{+}$ cells fail to suppress B cell differentiation indicating the presence of two functionally distinct subsets of $Gr1^{+}CD11B^{+}$ expressing cells. The antibody used to deplete $Gr1^{+}$ $CD11b^{+}$ cells *in vivo* in our studies depletes both $Gr1^{high}CD11b^{+}$ as well as $Gr1^{low}$ $CD11b^{+}$ cells, hence we cannot delineate the *in vivo* function of the two Gr1 expressing cell sub sets. *To examine the specific role the* $Gr1^{high}CD11b^{+}$ *and* $Gr1^{low}$ $CD11b^{+}$ *cells in vivo we have to develop a*

system where we can specifically deplete either of the cell population. It is to be noted that there is a lack of cell surface and morphological markers distinguishing the functional role of $Gr1^{high}CD11b^{+}$ and $Gr1^{low}$ $CD11b^{+}$ cells.

There are contrasting reports regarding the role of MDSC like cells in the pathogenesis of autoimmune diseases. The cell surface markers defining MDSC like cells (Gr1 and CD11b) are expressed by neutrophils, monocytes, and macrophages; these cell types are inflammatory in nature. The pro-inflammatory and disease promoting role of MDSC like cells in lupus-like disease development cannot be overlooked, but our studies show a protective role of MDSC like cells in lupus prone (NZBxNZW) F1 male mice. We also observed that $Gr1^{+}CD11b^{+}$ cells +acquire an inflammatory phenotype as the female (NZBxNZW) F1 male mice age. Hence, we speculate the existence of multiple cell subsets with in the $Gr1^{+}CD11b^{+}$ cells with specific functionality and regulatory properties. *The lack of cell surface markers and functional assays to determine smaller cell subsets within the $Gr1^{+}$ $CD11b^{+}$ cell population presents a challenge to distinguish between the immune-suppressive and inflammatory cell types.*

Our report is the first showing a direct immune-suppressive effect of $Gr1^{high}CD11b^{+}$ MDSC like cells on B cells, and that the activity is crucial to the role of $Gr1^{high}CD11b^{+}$ in mediating the progression of lupus-like disease in (NZBxNZW) F1 mice as B cells are the source of pathogenic antibodies. Also the elevated level of serum IL-10 after $Gr1^{+}$ cell depletion can contribute to the progression of lupus-like disease. The role of IL-10 in development of autoimmunity is debatable but it has been shown that IL-10 can promote B cell differentiation. *The source of IL-10 producing cells in the absence of $Gr1^{+}CD11b^{+}$*

is not known and further investigation is required to identify the cellular source of IL-10 in (NZBxNZW) F1 mice. We conducted studies described in Chapter 2 to examine the role of IL-10 as an immune-suppressive mechanism but our results indicate that IL-10 is not acting as an immunosuppressant. Finally, the protective role of testosterone in the development of lupus-like disease in (NZBxNZW) F1 has been well established since the 1970s but the underlying mechanisms are not clear. The regulation of immune-suppressive Gr1+ $CD11b^{+}$ cells *via* testosterone may explain, at least in part, the protective role of testosterone in (NZBxNZW) F1 mice.

Another interesting observation is that the $Gr1^{high}CD11b^{+}$ cells from female (NZBxNZW) F1 mice lose their suppressive ability post-pubertaly and hence female mice are unresponsive to *in vivo* depletion of Gr1+ cells as the treatments begin at 9-12 weeks of age. This time period coincides with the increase in female sex hormone (estrogen) levels and the beginning of disease progression, and chronic inflammation in female (NZBxNZW) F1 mice. The loss of suppression could be attributed to increased female sex hormones or the chronic inflammatory milieu present in female (NZBxNZW) F1 mice. As discussed in chapter 1, estrogen has an aggravating effect in driving lupus-like disease in (NZBxNZW) F1 mice and there is chronic inflammation going on in the form of elevated IL-6 and IFN-α level in female (NZBxNZW) F1 mice. We therefore cannot rule out either possibility, but it requires further experiments to firmly establish factors responsible for loss of suppression by $Gr1^{high}CD11b^{+}$ cells in post-pubertal female (NZBxNZW) F1 mice. We should also mention that the $Gr1^{low}$ $CD11b^{+}$ cells become stimulatory towards naïve B cells as the mice age which may be due to exposure to inflammatory cytokines like IFN-α and induction of NETs. Also the $Gr1^{low}$ $CD11b^{+}$ cells

are very close to low density granulocytes (LDGs) in cellular morphology and granular properties, and can further contribute to disease progression in older (NZBxNZW) F1 mice. It has been shown in human studies that NETs act as a source of auto-antigen for progression of autoimmunity. The loss of immune-suppressive function by female cells may also be attributed to the intrinsic genetic components of the female immune system. *Further studies are required to explore the loss of immune-suppressive ability of female cells. Reversing the loss of immune-suppression by female* $Gr1^{+}CD11b^{+}$ *cells may be an attractive target for therapy.* Our studies show that the number and function of $Gr1^{+}CD11b^{+}$ is differentially regulated in male and female lupus prone mice. These differences seem to be a combined effect of sex hormones as well as the genetic properties of the immune system.

The fact that female $Gr1^{high}CD11b^{+}$ cells use classical suppressive mechanisms (ROS and iNOS) associated with cancer MDSCs while the male $Gr1^{high}CD11b^{+}$ cells use an unknown suppressive mechanism indicates the intrinsic difference in the male and female $Gr1^{high}CD11b^{+}$ cells. We have conducted studies aimed at identifying the suppressive mechanism used my male $Gr1^{high}CD11b^{+}$ cells. One of the most promising candidate genes which were found to be upregulated in male $Gr1^{high}CD11b^{+}$ cells was S100A8/9 (unpublished data). There have been many studies defining the immune-regulatory role of S100A8/9 proteins on the function and accumulation of MDSCs in cancer models. *This difference may be attributed to the multigenetic nature of (NZBxNZW) F1 mouse model and needs further investigation. Moreover elucidating the suppressive mechanism used by male* $Gr1^{high}CD11b^{+}$ *cells may present novel therapeutic targets for manipulation in SLE.*

Another interesting observation is that the proportion of male (NZBxNZW) F1 developing lupus-like disease (~30%) resembles the proportion of male (NZBxNZW) F1 that have relatively lower levels of $Gr1^+$ $CD11b^+$ cells as compared to disease free male (NZBxNZW) F1 mice. This observation is potentially interesting as the level of these cells can be used as disease predicting bio-marker in susceptible individuals. There is a requirement of further studies to examine the possible correlation between $Gr1^+$ $CD11b^+$ cells and development of lupus-like disease in (NZBxNZW) F1 mice.

Since we used a T cell free system to test *in vitro* suppression of B cell differentiation, the next question we asked is whether the *in vivo* effect on antibody secretion is a direct effect of $Gr1^+CD11b^+$ cells on B cells or if it is mediated through T cells. To address this question we used an immunization approach (described in Chapter 3).

Here we show that female (NZBxNZW) F1 mice respond better to a T dependent immunization as compared to male (NZBxNZW) F1 mice. This is in line with the fact that female (NZBxNZW) F1 mice have a hyperactive immune response. Next we showed that depletion of Gr1+ cells only after immunization with a T cell dependent antigen lead to a substantially strong antigen-specific immune response in male (NZBxNZW) F1 mice. As observed earlier, the females did not respond to anti-Gr1 treatment. These findings suggested that *in vivo* suppression antibody production by Gr1+ cells is mediated through T cells. The enhanced antigen specific immune response was accompanied by elevated levels of PCs and GC B cells.

In chapter 1 we described that only $Gr1^{high}CD11b^+$ cells are capable of suppressing B cell differentiation *in vitro*; here we found out that only $Gr1^{low}CD11b^+$ cells were able to

suppress T cell proliferation *in vitro*. A similar loss in suppressive function of $Gr1^{low}CD11b^{+}$ cells was observed in the case of older females. Hence, we have established the specific suppressive role for the two separate Gr1 expressing populations in lupus-prone (NZBxNZW) F1 male mice. Additionally, both cell subsets lose their immune-suppressive capability in post-pubertal female (NZBxNZW) F1 mice.

Further, depletion of $Gr1^{+}$ cells lead to an increase in effector T cell subsets in immunized animals. As discussed in section 1.5, T_{FH} cells are essential for GC formation and generation of an antigen-specific antibody mediated immune response. We found that that $Gr1^{+}$ cell depletion leads to a rapid increase in T_{FH} cells *in vivo* after immunization and correlated with the increase in germinal center B cells. Indeed, we showed that $Gr1^{+}$ cells can suppress naïve T cell differentiation into T_{FH} cells. *Again the suppressive mechanism used by Gr1+ cells to suppress T_{FH} differentiation remains unknown and further investigation is required elucidate it.*

We can clearly state that testosterone regulated Gr1+ CD11b+ cells paly a protective role in lupus-prone (NZBxNZW) F1 male mice but they are functionally different in female (NZBxNZW) F1 mice. We can speculate that these cells have distinct inherent qualities and affect the progression of lupus-like disease in a different manner.

Genetics is one of the major factors contributing to the sex-bias observed in SLE and other autoimmune diseases. The hyperactive state of the female immune system and often X chromosome dosage are responsible for the female predominance in autoimmune disorders. The studies in Chapter 4 show that the power of female (NZBxNZW) F1 is enough to overcome the protective role of the testosterone driven immune-suppressive

$Gr1^+$ $CD11b^+$ population. Testosterone in intact male recipients was only able to mildly delay the onset of the disease without any effect on the end stage renal disease. The ability of the female hematopoietic cells to maintain high levels of lupus driving IFN-α irrespective of the hormonal environment of the host indicates the strong genetic pre-disposition of the female cells to drive lupus-like disease. In fact, we can speculate that the immune-suppressive $Gr1^+$ $CD11b^+$ cells can protect lupus prone male (NZBxNZW) F1 from disease progression due to the lack of a disease driving genetic program, as the females. *Additionally, identification of disease driving cell types in the female hematopoietic system can also provide therapeutic targets.*

To summarize, we can say that the sex-bias in lupus-like disease development in (NZBxNZW) F1 mice is an outcome of multiple factors. We have identified a testosterone driven $Gr1^+$ $CD11b^+$ cells immune-suppressive cell population which protects lupus prone male mice from disease. The female microenvironment comprising of female sex hormones as well as the pro-inflammatory cytokines may be responsible for fewer numbers and loss of suppression of $Gr1^+$ $CD11b^+$ cells. Also the differential function of $Gr1^+$ $CD11b^+$ cells indicates towards an intrinsic difference between male and female immune system. Also the BM chimera system shows the overpowering role of female immune system and genetics on the development of lupus-like disease.

Our studies open exciting avenues for therapeutic targets in terms of unknown immune-suppressive mechanism used by male $Gr1^+CD11b^+$ cells to regulate lupus like disease development in (NZBxNZW) F1 mice. Further, elucidation of testosterone mediated

upregulation of $Gr1^{+}CD11b^{+}$ cells and the use of $Gr1^{+}CD11b^{+}$ cell levels as a disease predictive bio-marker.

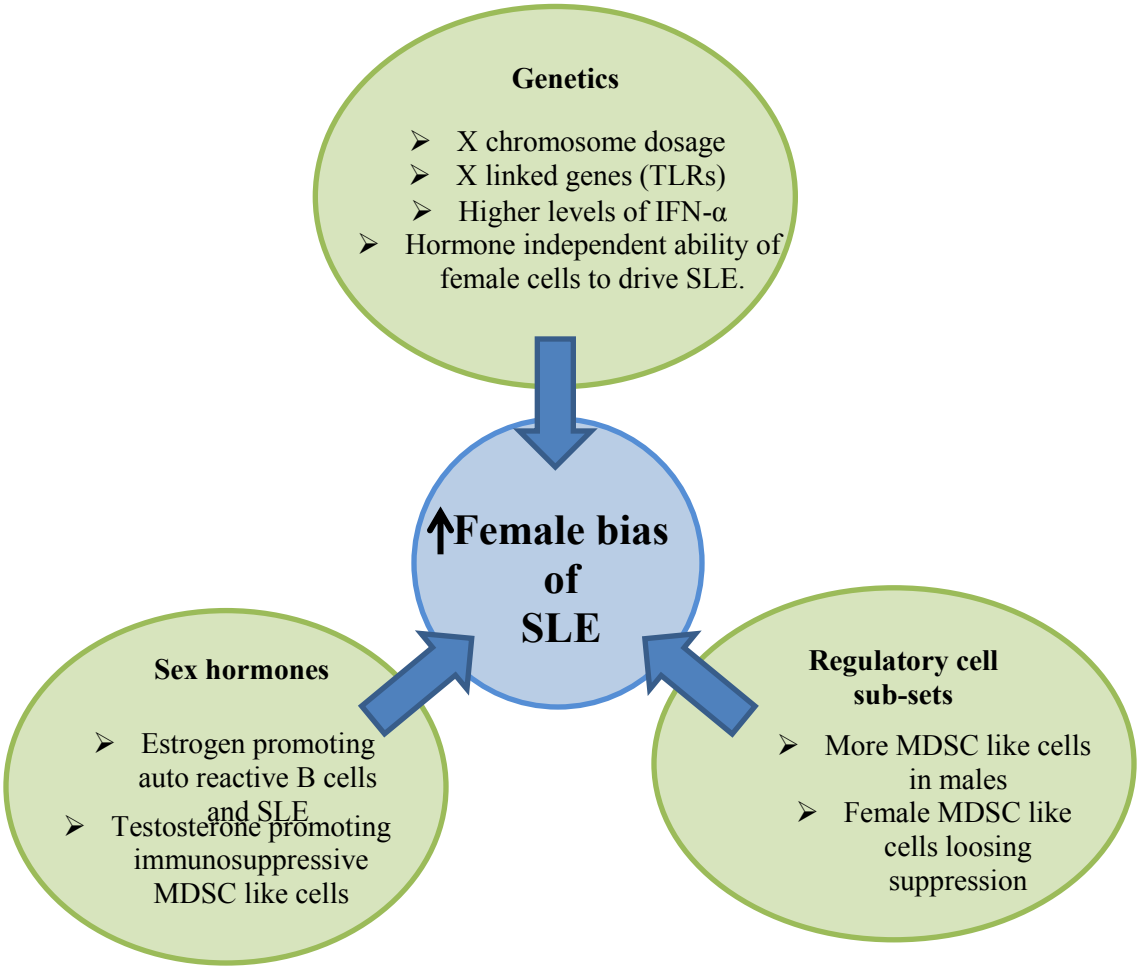

Figure 5.1 Relative contribution of different factors in sex-bias of SLE. The strong female sex-bias seen in SLE is the outcome of multiple factors working together. Genetics play an important role in the form of X linked gene expression and higher levels of disease promoting IFN-α production. Sex hormones play another crucial role as

estrogen promotes SLE and testosterone has an immune-suppressive function and protect from SLE. Lastly, we defined immune-suppressive MDSC like $Gr1^{+}$ cells upregulated in lupus prone male mice which can further explain the sex-bias observed in SLE.

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LIST OF PUBLICATIONS

1. **Abhishek Trigunaite**, Ayesha Khan, Evan Der, Sanjay Varikuti, and Trine N. Jørgensen. Gr-$1^{high}CD11b^{+}$ Cells Suppress B Cell Differentiation and Lupus-like Disease in Lupus-Prone Male Mice. ***Arthritis & Rheumatism*** 2013; 65 (9):2392-2402.

2. Iffland PH 2nd, Carvalho-Tavares J, **Trigunaite A**, Man S, Rasmussen P, Alexopoulos A, Ghosh C, Jørgensen TN, Janigro D. Intracellular and circulating neuronal antinuclear antibodies in human epilepsy. ***Neurobiology of Disease*** 2013; 59:206-219.

3. Der E, Dimo J, **Trigunaite A**, Jones J, Jørgensen TN. Gr1+ cells suppress antibody response to antigen challenge in (NZB x NZW) F1 male mice through inhibition of T follicular helper cells and germinal center formation. ***Journal of Immunol*ogy** 2014 Feb 15;192(4):1570-6).

4. A Davis, **A Trigunaite**, M K MacLeod, A C Johnson, P Marrack and T N Jørgensen. Intrinsic autoimmune capacities of hematopoietic cells from female New Zealand hybrid mice. ***Genes and Immunity*** 15, 153-161 (April/May 2014).

5. Evan Der, **Abhishek Trigunaite**, Ayesha Khan and Trine N. Jorgensen. Pro- and Anti-inflammatory Neutrophils in Lupus. **Journal of Clinical and Cellular Immunology.** doi: 10.4172/2155-9899.1000239.

6. **Abhishek Trigunaite**, Joana Dimo and Trine N. Jorgensen. Suppressive effects of androgens on immune system. **Cellular Immunology.** doi:10.1016/j.cellimm.2015.02.004.