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Role of B cell and hematopoietic cell intrinsic actions of ERα in lupus pathogenesis

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by

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Abstract

Role of B cell and hematopoietic cell intrinsic actions of ERα in lupus pathogenesis

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Lupus is a chronic autoimmune disease characterized by the presence of autoimmune B and T cells and the production of pathogenic antibodies against nuclear antigens. Lupus predominately affects women between menarche and menopause. There are both genetic and environmental risk factors which affect an individuals' risk of developing lupus. Estrogens are a risk factor for developing lupus and are thought to contribute significantly to the initiation and progression of disease. In lupus-prone mice, genetic knockout of a receptor for estrogen, estrogen receptor alpha (*ERα*), causes significant attenuation of lupus. Previous studies have not identified the cell type or types which mediate the effects of ERα on lupus. Estrogen has many effects on the immune system which could contribute to the development of autoimmunity in susceptible individuals. Particularly, estrogen promotes the survival of highly autoreactive B cells. Therefore, we hypothesized that ERα expression in hematopoietic cells promotes lupus, and more specifically, that ERα in B cells promotes lupus.

To test this hypothesis, we created two different murine models of lupus on the lupus-prone (NZB x NZW)F1 genetic background. To investigate the role of ERα in hematopoietic cells, we created chimeric mice with hematopoietic and non-hematopoietic cells with different *ERα* genotypes. Due to issues with the creation of successful chimeras, we were not able to use these mice to fully address our

hypothesis. However, these studies revealed that estrogen plays a role in the success of hematopoietic reconstitution in females.

To address the hypothesis that *ERα* expression in B cells promotes lupus, we created a (NZB x NZW)F1 model with B cell specific deletion of *ERα*. Although only a moderate proportion of B cells had successful deletion of *ERα*, this was sufficient to cause a significant attenuation of lupus. Mice with B cell specific *ERα* deletion had fewer activated B cells, produced fewer pathogenic autoantibodies, and had significantly prolonged survival compared to control mice. Therefore, these studies have shown that *ERα* expression in B cells promotes lupus in the (NZB x NZW)F1 model of lupus.

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List of Abbreviations

Abbreviation	Definition
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GPER	G protein-coupled estrogen receptor 1
AF	Activation factor
DBD	DNA binding domain
ERE	Estrogen response element
HD	Hinge domain
LBD	Ligand binding domain
HRT	Hormone replacement therapy
HSC	Hematopoietic stem cell
MPP	Multipotent progenitor
Ig	Immunoglobulin
Treg	T regulatory cell
PPT	Propyl pyrazole triol
SLE	Systemic lupus erythematosus
ACR	American College of Rheumatology
dsDNA	Double stranded DNA
ANA	Anti-nuclear antibodies
BAFF	B cell-activating factor
Breg	B regulatory cell
BCR	B cell receptor
NSAID	Non-steroidal anti-inflammatory
FDA	Food and Drug Administration
BAFF	B-cell activating factor
SERM	Selective estrogen receptor modifier
TSEC	Tissue selective estrogen complex

Chapter 1: Introduction

Estrogens

Estrogens are a group of naturally occurring and synthetic steroid hormones which regulate the female reproductive system and are responsible for the development of female secondary sex characteristics. There are three kinds of naturally occurring estrogens, which all have different biological activity: estrone (E1), estradiol (E2), and estriol (E3). Estrogens are produced from cholesterol through aromatization of androgens. In non-pregnant premenopausal women, the ovary is the principle source of estrogens E1 and E2, but small amounts of estrogen are also produced in adipose tissue, skin, bone, and brain, and in men estrogens are produced in the testes (reviewed in Simpson, 1999). E3 is produced by modification of E1 or E2 and is primarily produced in the placenta during pregnancy. Serum E2 and E1 increase in the late follicular stage of the menstrual cycle, while the levels of E3 are generally low and do not fluctuate with the menstrual cycle (Lipsett, 1978). In premenopausal women, the average serum E2 level varies from about 50 pg/ml to 125 pg/ml over the course of the menstrual cycle, and postmenopausal women have an average of 54 pg/ml serum E2 (Ghosh, 2014). Men have an average serum E2 level of 43 pg/ml (Travison, 2014). Estrogens have many physiological effects in both women and in men, which are discussed in more detail below.

As steroid hormones, estrogens diffuse freely across cell membranes and elicit genomic and non-genomic effects by binding to estrogen receptors (ERs). E2 is the most potent of these naturally occurring estrogens because of its superior binding affinity for ERs. The relative binding affinity of these naturally occurring estrogens to receptors is E2 > E1 > E3 (Korenman, 1968).

Estrogen Receptors

Estrogens signal through estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and membrane-bound G protein-coupled estrogen receptor 1 (GPER). Estrogens bound to GPER cause rapid calcium mobilization, and signaling through GPER is responsible for some of the rapid effects of estrogen. Estrogens bound to nuclear hormone receptors ERα and/or ERβ cause transcriptional effects on target genes. This introduction will focus on ERα and ERβ.

ERα and ERβ are nuclear hormone receptors found most abundantly in the cell nucleus. The most abundant ERα in the nucleus is the full length 66 kDa protein. In the nucleus, ERα and ERβ can act as transcription factors or interact with other transcription factors to impact gene expression. Both ERα and ERβ can also be found in the cytoplasm and bound to the plasma membrane. Upon ligand binding, ERs in the cytoplasm change conformation to reveal a nuclear localization signal and then translocate to the nucleus. Plasma membrane ERα is relatively rare, and association of this 46 kDa splice variant of ERα with the plasma membrane is dependent on post-translational palmitoylation (Li, 2003). Palmitoylated ERβ is also found in the plasma membrane (Galluzzo, 2007). Plasma membrane bound ERα and ERβ are responsible for the rapid effects of estrogen signaling not caused by GPER. Estrogen signaling through plasma membrane ERα leads to phosphorylation of AKT and ERK (Pedram, 2014). ERβ is also found in the mitochondria where it increases the threshold for apoptosis in a ligand-independent manner (Yang, 2004; Liang, 2015).

The *ERα* and *ERβ* genes are composed of analogous functional domains A-F which have varying degrees of homology (Figure 1.1). Both genes have an N-terminal A/B domain which contains an activation factor (AF)-1 transcriptional regulation domain which is not well conserved between *ERα* and *ERβ*. Next, there is domain C, which

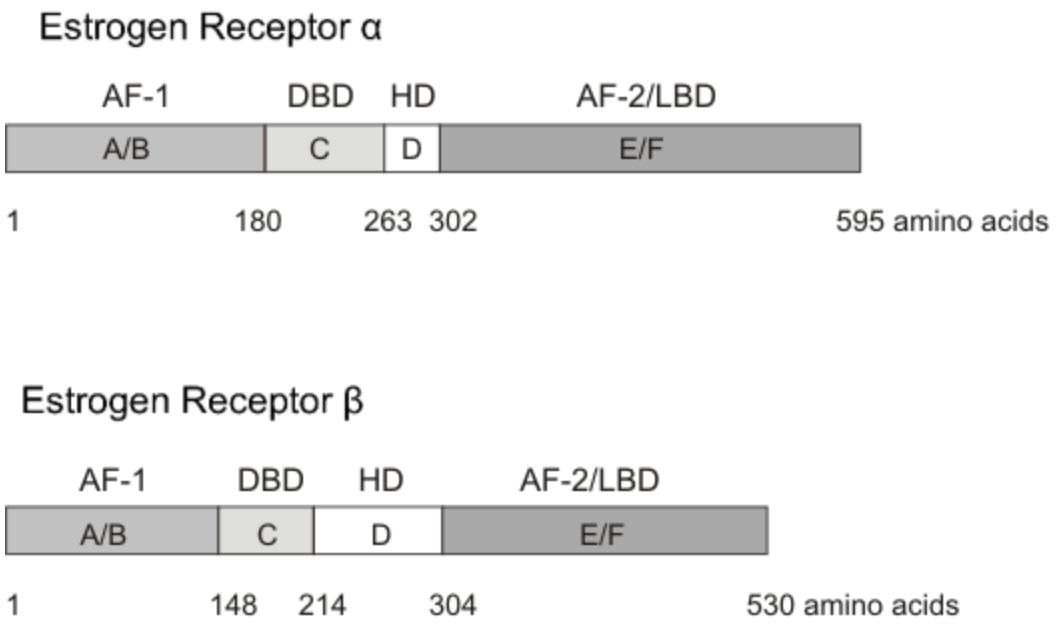

Figure 1.1 **Functional domains of ERα and ERβ**

Figure 1.1 **Functional domains of ERα and ERβ**

ERα and ERβ are composed of analogous functional domains. The A/B domain contains an activation function (AF) domain, AF-1, which regulates the transcriptional activity of the ER. Domain C contains the DNA binding domain (DBD). The D domain contains the hinge domain (HD) as well as a nuclear localization signal which is revealed upon ligand binding. The E/F domain contains a second AF binding site (AF-2) and the ligand binding domain (LBD).

contains the DNA binding domain (DBD) and dimerization domain. This domain is well conserved between *ERα* and *ERβ* and allows both receptors to bind estrogen response elements (EREs) in the genome. Domain D is also involved in dimerization, as well as binding of heat shock proteins, and contains the hinge domain (HD), which undergoes a conformational change to reveal the nuclear localization signal upon ligand binding. The C-terminal E/F domain contains a ligand binding domain (LBD) which forms a hydrophobic pocket, and the AF-2 transcriptional regulation domain. There is a moderate degree of homology in the ligand binding domain, which causes *ERα* and *ERβ* to have different binding affinities for different estrogenic ligands. Variation in the AF domains and ligand binding domain causes ERα and ERβ to have different transcriptional effects.

In the inactive state, ERα and ERβ are bound to heat shock proteins, which are released upon ligand binding. After ligand binding, ERα and ERβ form homo- or heterodimers through interaction of peptides in the dimerization domain. Homodimers of ERα and ERβ have different physiological activities, while heterodimers act like ERα homodimers (Li, 2004). The ER dimer moves to the nucleus where it acts as a transcription factor, either by binding DNA and acting as a transcription factor in ERE-dependent signaling, or by binding other transcription factors and acting as part of a transcription factor complex without directly binding DNA in ERE-independent signaling. The conformation of the ER dimer is independently affected by both the ligand and the sequence of the ERE site with which it interacts (Yi, 2002; Ramsey, 2001). The ligand, and not the ERE sequence, dictates the recruitment of co-factors such as SRC-1, TIF-1, TIF-2, and AIB-1 to the ER complex (Yi, 2002).

The ERE consensus sequence is a 13 base pair palindrome, GGTCAnnnTGACC, which can contain up to two deviations from the consensus sequence and still elicit binding, although multiple nearby EREs are required for efficient binding of imperfect sequences

(Martinez, 1989). ERα has an approximately two-fold greater affinity for the same EREs as ERβ, which could be a mechanism underlying the different transcriptional activity of these receptors (Yi, 2002). Approximately 70,000 potential ERE sites have been found in the human and mouse genomes, and the most highly estrogen-responsive genes contain multiple EREs in their promoters (Bourdeau, 2004; Martinez, 1989; Sathya, 1997).

Estrogen Receptors and Reproduction

ERα and ERβ are found throughout the reproductive system in the ovaries, uterus, and mammary glands in females and the testis of males in both humans and mice (Pelletier, 2000; Irsik, 2013). ERα signaling plays an essential role in regulation of hormones and reproduction. In mice with *ERα* knockout (*ERα*$^{-/-}$) both female and male mice are infertile. Female *ERα*$^{-/-}$ mice have abnormal physiology of the ovaries and uterus; the ovaries develop large hemorrhagic cysts and do not release mature follicles, while the fallopian tubes and uterus are small and the uterus does not increase in size in response to E2 treatment (Dupont, 2000; Lubahn, 1993). Infertile male *ERα*$^{-/-}$ mice have defects in spermatogenesis resulting in significantly reduced sperm number as well as defects in the motility of the few sperm that are produced (Eddy, 1996). Interestingly, it is somatic cells, not germ cells, which require ERα signaling for normal sperm production and male fertility (Mahato, 2001). Global heterozygosity for *ERα* (*ERα*$^{+/-}$) results in normal fertility in both females and males. Therefore, at least one functional copy of *ERα* is essential for normal fertility. In addition to loss of fertility and physiological abnormalities, *ERα*$^{-/-}$ mice also have hormonal abnormalities. Female *ERα*$^{-/-}$ mice have a ten-fold increase in serum E2 and an increase in serum testosterone of a similar magnitude (Eddy, 1996). Serum testosterone levels are elevated by two-fold in male *ERα*$^{-/-}$ mice, but E2 levels are not affected (Eddy, 1996; Parikka, 2005). The increased serum hormone levels in *ERα*$^{-/-}$

mice are the result of lack of feedback in the hypothalamic-pituitary-gonadal axis. Therefore, although *ERα*$^{-/-}$ mice can provide some useful information, it is important to remember that in this system estrogen and testosterone levels are significantly elevated in females, and signaling through ERβ, GPER, and androgen receptors may be impacted by these hormonal changes. To study the effects of ERα in specific estrogen-sensitive cells and tissues, models with conditional activation or deletion of *ERα* have been developed. Using a conditional deletion approach, it has been shown that *ERα*$^{-/-}$ gonadotropin α-subunit- expressing pituitary cells are sufficient to cause infertility in female mice, but not in males (Gieske, 2008). These females produce normal follicles, not the hemorrhagic cysts observed in global *ERα*$^{-/-}$ females, but do not have regular estrus cycles (Gieske 2008). Fertility in female mice requires *ERα* expression in multiple tissues, for instance, in gonadotropin α-subunit-expressing pituitary cells for regulation of estrus cycles, and in the uterus for estrogen-induced growth of the uterine lining.

Like *ERα*, *ERβ* is widely expressed in the female and male reproductive tracts. However, unlike *ERα*$^{-/-}$ mice, *ERβ* null (*ERβ*$^{-/-}$) mice are not completely sterile. *ERβ*$^{-/-}$ males have normal fertility, and *ERβ*$^{-/-}$ females have reduced fertility with fewer litters and fewer pups per litter than mice with normal *ERβ* expression (Krege, 1998). The uterus of *ERβ*$^{-/-}$ females appears normal and is responsive to estrogen, but the ovaries release significantly fewer follicles than normal (Krege, 1998). Thus, *ERβ* is not essential for reproduction, but does affect fertility in females.

Unsurprisingly, mice with combined *ERα* and *ERβ* deficiency are completely sterile (Couse, 1999). Individually, *ERα*$^{+/-}$ and *ERβ*$^{-/-}$ do not cause complete infertility in mice of either sex, so it is not unexpected that *ERα*$^{+/-}$ *ERβ*$^{-/-}$ male mice have normal fertility, however, it was surprising that *ERα*$^{+/-}$ *ERβ*$^{-/-}$ female mice are unable to produce mature ovarian follicles and are completely sterile (Dupont, 2000). These data suggest that both

ERα and *ERβ* are important for female fertility and that there is some functional redundancy between these receptors. Additionally, *ERα* significantly affects male fertility while *ERβ* does not play an essential role.

The expression level of ERs, as well as polymorphisms in these genes, impacts human fertility. Sperm from men with varicocele-related infertility express lower levels of *ERα* and *ERβ* than healthy sperm, which leads to reduced estrogen-induced motility (Guido, 2011). Polymorphisms in *ERα* and *ERβ* in both women and men are associated with differences in fertility potential (Corbo, 2007; Zulli, 2010). Clearly, estrogen signaling through ERα and ERβ plays an important role in reproduction.

Estrogen Receptors in Non-Reproductive Physiology

ERs are not only important in reproduction, but also in other tissues throughout the body. Although ERs are expressed by both females and males, females often have higher levels of ER expression, and the potential magnitude of ER signaling is greater due to an increased amount of estrogen available as a ligand. *ERα* and *ERβ* are expressed in the kidney, liver, and heart in both female and male mice, although in the kidney males only produce 25% the ERβ as females, and ERα is produced at a lower level by males in all of these tissues (70%, 6%, and 1% of female level, respectively) (Irsik, 2013). In the kidney, ERα regulates urine osmolality and water homeostasis (Cheema, 2015).

In the cardiac system, estrogens are protective against cardiac disease. Women who receive postmenopausal hormone replacement therapy (HRT, estrogen alone or estrogen and progestin) within 10 years of menopause have a reduced risk of death from coronary heart disease, although this same protection was not seen in women who began treatment more than 10 years after menopause (Boardman, 2015). ERα in the coronary endothelium is protective against ischemia-related loss of acetylcholine

responsiveness and reduces the size of the damaged area after myocardial infarct (Favre, 2010). ERα also plays an important role in E2-mediated protection from the development of atherosclerosis, in part by reducing cholesterol levels (Hodgin, 2001). In the brain, both *ERα* and *ERβ* are widely expressed, and both contribute to the prevention of hypertension by acting on different regions of the brain (reviewed in Hay, 2014).

Many of the cells involved in the growth and maintenance of bones express *ERα*. Loss of *ERα* results in bone defects. $ERα^{-/-}$ mice have shorter vertebrae and limb bones than $ERα^{+/+}$ mice, increased trabecular bone volume, and increased tibial thickness (Parikka, 2005). At the cellular level, osteoblasts from $ERα^{-/-}$ mice produce less type I collagen, and female $ERα^{-/-}$ mice have fewer osteoclasts compared to $ERα^{+/+}$ mice (Parikka, 2005). ERα not only plays an important role in bone growth, but also bone remodeling. Many of the cell types involved in maintaining bone structure express *ERα*, and estrogens play an important role in maintaining bone mineral density and preventing the development of osteoporosis. HRT in post-menopausal women prevents osteoporosis-associated bone loss and fracture (Cauley, 2003). In addition to regulating bone growth and remodeling, ER signaling also plays an important role in the bone marrow by regulating hematopoiesis.

Estrogen and the Immune System

The immune systems of women and men differ intrinsically, which has long been attributed the higher levels of estrogen in women. These intrinsic differences are thought to be related to pregnancy and the need to both immunologically tolerate a fetus and protect the body from external pathogens. Women produce more robust cellular and humoral immune responses than men do, and consequently are at a higher risk of developing autoimmune diseases. Estrogen does not cause an immune response, but

instead enhances humoral immune responses (Carlsten, 1989). Hematopoietic stem cells (HSCs) are the source of immune cells, and estrogen has far-reaching effects on the whole immune system by acting on these undifferentiated cells. Both HSCs and the more differentiated multipotent progenitors (MPPs) express *ERα*, while *ERβ* expression is only detected in HSCs (Sanchez-Aguilera, 2014). There is no difference in the number or frequency of HSCs or MPPs in the bone marrow of females and males (Nakada, 2014). In $ERα^{+/+}$ females, both HSCs and MPPs divide more frequently than in $ERα^{+/+}$ males or $ER^{-/-}$ mice of either sex, which all divide at the same lower rate (Nakada, 2014). When estrogen levels are increased in females by pregnancy or E2 administration, the frequency of HSC division increases further, and the number of HSCs in the bone marrow significantly increases (Nakada, 2014, Illing, 2012). Males also experience increased HSC division when treated with E2, propyl pyrazole triol (PPT, an ERα agonist), or tamoxifen (an ERα agonist in hematopoietic cells) (Nakada, 2014; Sanchez-Aguilera, 2014; Thurmond, 2000). Ergo, females naturally have an increased rate of HSC division compared to males, which is dependent on ERα signaling and is due to a naturally higher serum level of E2.

Although HSCs in female mice naturally divide more rapidly than in males, supra-physiological levels of division may have detrimental effects on the HSC population. Treatment with high dose tamoxifen significantly decreases the long term HSC, short term HSC, and MPP populations, while increasing the cycling of normally quiescent long term HSCs (Sanchez-Aguilera, 2014). After three rounds of serial transplantation, E2 treated HSCs have a reduced ability to reconstitute the HSC population in the bone marrow compared to untreated cells (Illing, 2012). Estrogens lead to an increased rate of HSC division, and at high enough levels to exhaustion of the HSC population.

Although there is strong evidence that *ERα* expression in HSCs plays an important role in regulating HSC division, it is not the only way through which estrogens affect the HSC population. Estrogen signaling via other receptors, as well as ERα signaling in stromal cells, can also affect HSC division. In one study, *ERα* knockout mice treated with a high dose of E2 had significant reduction in bone marrow HSCs, although this was not as dramatic as was seen in $ERα^{+/+}$ mice (-0.3 vs -0.8 fold) (Thurmond, 2000). This suggests that ERα only partially regulates this effect, and that signaling through ERβ or GPER may be involved. The same study also found that expression of *ERα* in both the hematopoietic and non-hematopoietic cells contributed to the decrease in the number of HSCs caused by high dose E2 (Thurmond, 2000). In short, estrogens promote the division of HSCs, which is partially mediated by ERα. Supra-physiological amounts of estrogen can cause exhaustion of the HSC population which, in turn, can lead to alterations in the immune system.

In addition to their effects on HSCs, estrogens also impact the differentiation of lymphocytes (see figure 1.2 for an overview of B cell development). Estrogen causes an overall decrease in the total B cell population in the bone marrow; more specifically, it causes a decrease in the number of developing B cells, and an increase in the proportion of mature bone marrow B cells (Medina, 2001; Thurmond, 2000; Erlandsson, 2002). Additionally, estrogen causes a decrease in the number of B cells in the spleen (Erlandsson, 2002). These effects on B cell populations are likely caused by ER regulation of multiple genes. Estrogen signaling increases the expression of *Bcl-2*, an anti-apoptotic molecule, in developing and mature B cells; increased Bcl-2 causes an increase in mature B cells in the bone marrow (Grimaldi, 2002; Strasser, 1991). However, overexpression of *Bcl-2* does not affect the proportion of developing B cells in the bone marrow or mature B cells in the spleen (Strasser, 1991). Expression of *sFRP5*,

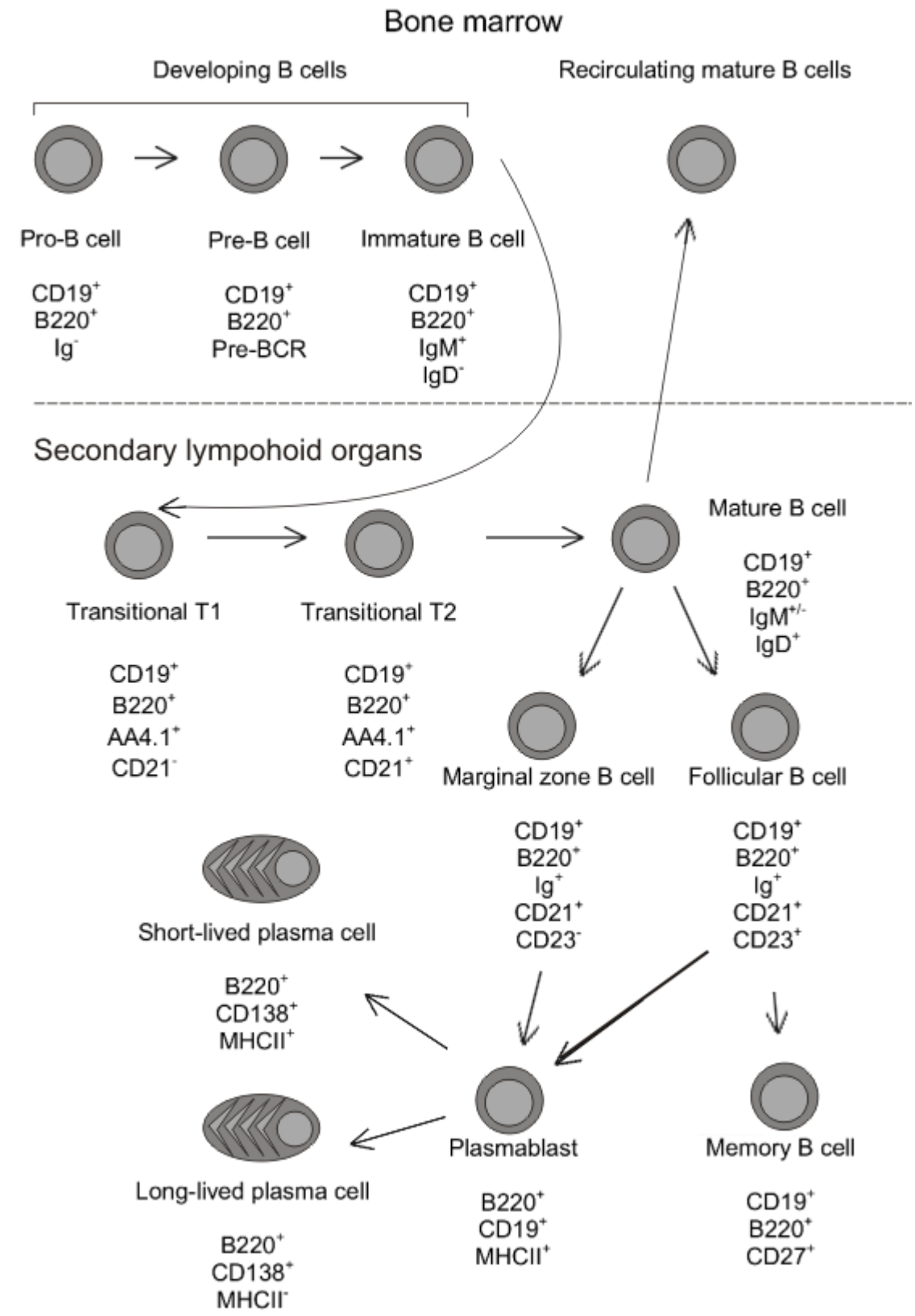

Figure 1.2 **B cell development**

Figure 1.2 **B cell development**

B cells originate in the bone marrow from hematopoietic stem cells (HSCs) which differentiate into multipotent progenitors (MPPs), and then common lymphoid progenitors (CLPs) (not shown). CLPs differentiate into pro-B cells which undergo immunoglobulin (Ig) heavy chain VDJ recombination. At the pre-B cell stage cells express the recombined heavy chain with surrogate light chain forming the pre-BCR. Heavy chain autoreactivity is mediated by additional rearrangement, and cells with autoreactive heavy chain specificity which are not successfully remediated die via apoptosis. Pre-B cells undergo Ig light chain VJ recombination. Immature B cells express both heavy and light chains as IgM, and autoreactive cells are remediated or are rendered anergic. Immature B cells leave the bone marrow and travel to secondary lymphoid organs, such as the spleen. In secondary lymphoid organs Transitional T1 B cells must undergo another checkpoint for autoimmunity before becoming Transitional T2 cells, and then mature B cells. Mature B cells express IgD and can become marginal zone or follicular B cells. Upon stimulation with cognate antigen and T cell help, memory B cells and plasmablasts are produced. Plasmablasts give rise to long-lived and short-lived plasma cells.

a modulator of Wnt signaling, is also increased by estrogen, and causes a decrease in developing B cell populations in bone marrow and total B cell population in the spleen (Yokota, 2015). These data suggest that estrogen causes a reduction in developing B cells and fewer splenic B cells by regulating the expression of multiple genes. Both ERα and ERβ are involved in the estrogen-mediated decrease in total bone marrow B cells and the increased proportion of mature B cells in the bone marrow (Erlandsson, 2003). The B cells of pre- and post-menopausal women and men all express *ERα* and *ERβ* at approximately the same level, but it is the amount of estrogen (and not testosterone) in the serum that negatively correlates with the total number of bone marrow B cells (Phiel, 2005; Erben, 2001). Additionally, estrogen promotes polyclonal B cell activation, which results in an increased number of antibody-producing cells, and increased serum immunoglobulins (Igs) (Carlsten, 1992; Nikolaevich, 1991). There are many ways in which estrogen promotes the development of autoreactive B cells, which will be discussed later.

In addition to its effects on B cell development, ERα plays an important role in the development of the thymus and thymic T cell populations. Both $CD4^+$ and $CD8^+$ T cells express *ERα* and *ERβ* at levels which are consistent among premenopausal women, postmenopausal women, and men (Phiel, 2005). Estrogen, acting through ERα, negatively regulates an early lymphocyte precursor population which gives rise to T cells, and causes decreased thymus cellularity (Medina, 2001; Islander, 2003). In the thymus, estrogen also causes a decrease in the immature double positive ($CD4^+CD8^+$) T cell population and an increase in more mature single positive ($CD4^+$ or $CD8^+$) T cells (Staples, 1999). This shift in T cell populations is also observed during pregnancy when estrogen levels are naturally increased (Rijhsinghani, 1996). In a competitive repopulation assay, E2-treated lymphocyte precursors produced fewer T cells than

untreated precursors, but the T cells differentiated into single and double positive T cells at normal ratios (Medina, 2001). Similarly, the pregnancy-induced disruption in thymic T cell populations normalizes postpartum and thymic cellularity returns to normal after weaning (Rijhsinghai, 1996).

The effects of estrogen on T cell development are mediated through both ERα and ERβ. *ERα*$^{-/-}$ mice have significantly smaller thymi with lower cellularity compared to *ERα*$^{+/+}$ mice (Staples, 1999). For normal size and cellularity of the thymus, expression of *ERα* is needed in both the stromal and hematopoietic cells (Staples, 1999). E2 treatment in *ERα*$^{+/+}$ *ERβ*$^{+/+}$, *ERα*$^{-/-}$, and *ERβ*$^{-/-}$ mice causes a decrease in thymic cellularity, which indicates that neither ERα nor ERβ alone is the sole mediator of the effects of E2 on thymus cellularity (Staples, 1999; Erlandsson, 2001). Thymus structure and T cell development are regulated by estrogen in a complex way not entirely dependent on either ERα or ERβ, and the effects on thymic cellularity and T cell populations are reversible when estrogen levels return to normal.

Estrogen also affects the immune response by regulating the balance of mature T cell populations. A balance among IFNγ-, IL-2-, and TNFα-producing Th1 cells that promote a cell-mediated immune response and IL-4-, IL-6-, and IL-10-producing Th2 cells that promote a humoral immune response is necessary to prevent inappropriate immune responses. Estrogens affect the Th1/Th2 balance in a bimodal manner, with low levels leading to an increased Th1 response and high levels leading to a Th2 response. Studies have shown that this effect is dependent on the expression of *ERα* in hematopoietic cells, specifically in T cells (Maret, 2003; Lelu, 2011).

Th17 cells are CD4^{+} T cells that secrete pro-inflammatory IL-17 cytokines, and thus play an important role in inflammatory and autoimmune diseases. Depending upon the

stimulation conditions, estrogen can promote or suppress the production of IL-17 (Khan, 2010; Lelu, 2011). Similarly, estrogen has different effects on Th17 cells in different disease models. Estrogen increases the number of IL-17-secreting cells and the amount of IL-17 produced in lupus-prone (NZB x NZW)F1 mice, consistent with estrogen's pathogenic role in lupus (Khan, 2010). In contrast, in a murine model of arthritis, estrogen signaling through ERα plays a protective role by restricting the localization of Th17 cells so that they do not reside in inflamed joints (Andersson, 2015).

T regulatory cells (Tregs) promote self-tolerance in the periphery by inhibiting the function of effector T cells. Increased levels of estrogen, by both E2 administration and pregnancy, cause an increase in the population of Tregs via ERα signaling (Polanczyk, 2004). Not only does E2 cause an increase in the number of Tregs, but it also increases their capacity to suppress effector T cells (Prieto, 2006).

Estrogen signaling, through ERα, can affect mature T cell populations in a number of important ways, including regulating the balance of Th1/Th2 cells, regulating Th17 cell activity, and increasing the regulatory capabilities of Tregs. Unlike with B cells, for which estrogens are known to promote the development of autoreactive cells, the effect of estrogens on the development of autoreactive T cells has not been studied.

In addition to its effects on lymphocytes, estrogen may also impact the differentiation and function of myeloid cells. *ERα* is expressed in myeloid lineage cells (Sanchez-Aguilera, 2014). However, reports vary on the effect of estrogens on the differentiation of myeloid cells. One group found that the differentiation potential of common myeloid progenitors is not affected by E2 treatment, while another reported that HSCs are less capable of differentiating into granulocytes after treatment with E2 (Medina, 2001; Illing, 2012). ERα signaling can either inhibit or promote the differentiation of dendritic cells,

depending on the cytokine environment, but it always enhances the dendritic cells' pro-inflammatory capacities (reviewed in Kovats, 2012; Seillet, 2013). Megakaryocyte and platelet counts are low when the E2 level is low and high when E2 levels are high (Fox, 2006). There is not a consensus about the effects of estrogens on monocyte function. One recent study showed that human primary monocytes have attenuated production of pro-inflammatory cytokines upon treatment with E2, while another found that women have higher levels of activated monocytes compared to men (Pelekanou, 2016; Jiang, 2014). Estrogen, through macrophage-intrinsic expression of *ERα*, promotes macrophage activation and pro-inflammatory cytokine production (Calippe, 2010). Clearly, estrogen signaling affects myeloid cells in complex ways, sometimes promoting and sometimes suppressing the immune response. More research into the effects of estrogen on different myeloid lineage cells is needed to form a more complete understanding of these effects.

As discussed here, in addition to their role in reproduction, ERs have a wide range of effects on normal physiological processes throughout the body, including a strong impact on the development and function of the immune system. Women are more likely to develop most autoimmune diseases than men. This phenomenon is thought to be due to intrinsic, estrogen-mediated, differences in the immune system. In order to develop improved therapies for estrogen-mediated diseases, we must increase our understanding of the role that estrogen signaling plays in different cells involved in the pathogenesis of these diseases.

Systemic Lupus Erythematosus

Many diseases have a strong sex bias. Nearly all autoimmune diseases have a female gender bias, including Hashimoto's thyroiditis, Sjogren's syndrome, rheumatoid arthritis, and multiple sclerosis. One autoimmune disease with particularly strong sex bias is

Systemic Lupus Erythematosus (SLE or lupus); between 90-95% of SLE patients are women. The prevalence of SLE is approximately 107-150 per 100,000 adults, and 180-250 per 100,000 women (Chakravarty, 2007). SLE is the most common and the most severe form of lupus. SLE is a heterogeneous disease that can impact any organ system and cause a variety of symptoms. Patients that have 4 or more of the 11 symptoms defined by the American College of Rheumatology (ACR) are diagnosed with SLE. These symptoms include malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, kidney disorder, neurological disorder, blood disorder, immunologic disorder, and abnormal antinuclear antibody (Arthritis and Rheumatism, 1999). Lupus is a dynamic disease and lupus patients experience alternating periods of flare and remission.

Although there is great diversity in the symptoms that lupus patients can develop, there are many immunological alterations, including the presence of autoreactive B and T cells, which are commonly observed in lupus patients. The defining characteristic of lupus is the production of antibodies against nuclear antigens– particularly the production of pathogenic double stranded DNA (dsDNA) antibodies. When these dsDNA autoantibodies bind antigen they form immune complexes which are deposited in tissues and cause an inflammatory immune response and tissue damage. Other common immune abnormalities seen in lupus patients include aberrant B and T cell activation, defective clearance of apoptotic debris, and production of cytokines that promote humoral and inflammatory immune responses.

Currently, the etiology of lupus is unknown, but it is thought to be caused by a combination of genetic and environmental factors. Studies examining the incidence of lupus in monozygotic and dizygotic twins have shown that monozygotic twins are more likely to both have lupus than dizygotic twins, but that there is not complete concordance

(Deapen, 1992). In addition to having a twin with lupus, having first degree relatives with lupus significantly increases an individual's risk of lupus. Those with one first degree relative with lupus have a relative risk of 17.04, which increases to 35.09 for someone with two first degree relatives with lupus (Kuo, 2015). Additionally, many genes associated with increased risk of lupus have been identified in lupus patients (Chung, 2014; Radanova, 2015). These studies have clearly established that there is a genetic component to lupus, but lupus is not a purely genetic disease.

It is believed that genetics predispose an individual to develop lupus and environmental factors trigger disease onset through phenomena like epitope spreading or molecular mimicry. Although there are likely many different initiating circumstances which can prompt the development of lupus in susceptible individuals, there is evidence that infections are a likely trigger of autoimmunity. Infection provides an opportunity for the immune system to experience epitope spreading, superantigen exposure, and molecular mimicry in an activated state. There have been reports of patients that experience an infection shortly before developing symptoms of lupus (Yamazaki, 2015; Perez-Mercado, 2010; Rajadhyaksha, 2012). By a completely different mechanism, a theory known as the hygiene hypothesis postulates that infection by microbes is protective against autoimmunity, and that lack of infection causes the immune system to develop abnormally and react against the self. So far, there is some support for this hypothesis in the form of a few case studies, and the assertion that people of African ethnicity living in the western world develop lupus at higher rates than Africans living in Africa, where they presumably experience more infections (Praprotnik, 2008; Bae, 1998). However, this assertion may be incorrect as similar rates of antinuclear autoantibody production have been found in African Americans and Africans of the same ancestral heritage (Gilkeson,

2011). Instead, the idea lupus is less common in Africa may be due to differences in the diagnostic abilities and availability of health care in western and African countries

Although the etiology of lupus is unknown, a defining characteristic of this disease is the production of anti-nuclear antibodies (ANA), including antibodies against dsDNA. Auto-antibodies are produced by activated autoreactive B cells that either escape negative selection at developmental checkpoints or are produced by somatic hypermutation (SHM) in the periphery. ANA can be detected in 95% of lupus patients of African-American, Afro-Caribbean, and Caucasian heritage (Somers, 2014; Flower, 2012). Antibodies specific to dsDNA are only detected in only about 37% of lupus patients (Hanly, 2016, Wichainun, 2013). Detection of both ANA and anti-dsDNA antibodies is highly specific for lupus (88-96%, and 97-100% respectively), although ANA are occasionally found in healthy controls (8%) and both antibodies are occasionally found in patients with multiple other health problems (12% and 3%, respectively) (Wichainun, 2013). Although not detectable in all lupus patients, anti-dsDNA antibodies are very specific to lupus.

In addition to autoreactive B cells and autoantibody producing plasma cells, lupus patients have other immune abnormalities that contribute to disease. They have significantly higher levels of B cell-activating factor (BAFF), which is secreted by monocytes and activated T cells and promotes the maturation of B cells to plasma cells (Elbirt, 2014). Patients with lupus have more plasmocytes and plasmablasts than healthy controls (Korganow, 2010). Most B cells from lupus patients express less *CD19* than controls, which may prevent the negative selection of autoreactive B cells (Korganow, 2010). Some lupus patients have a pre-plasma B cell population with high *CD19* expression, which is associated with adverse long-term clinical outcomes (Culton, 2007; Nicholas, 2008). It is thought that these $CD19^{hi}$ B cells are easily activated and are

autoreactive. Regulatory B cells (Bregs) are IL-10-producing B cells which negatively regulate the inflammatory immune response. Bregs from lupus patients cannot effectively inhibit T cell proliferation (Gao, 2014). In a murine lupus model that lacks mature B cells, lupus is completely attenuated (Shlomchik, 1994). In the same murine lupus model, when mature B cells are present but cannot secrete antibodies, some, but not all, lupus-related outcomes are improved (Chan, 1999). These studies point to both antibody-dependent and antibody-independent roles for B cells in lupus.

Like B cells, lupus patients also have autoreactive T cells that recognize nuclear antigens. T cells from lupus patients are resistant to activation-induced apoptosis, which leads to the survival of autoreactive T cells (Kim, 2010). The T cells of lupus patients also have more rapid signaling than healthy individuals due to lower TCRζ levels (Liossis, 1998). When activated, autoreactive B and T cells cause an inflammatory immune response which leads to tissue damage. Normally, nuclear proteins are sequestered and are not available to autoreactive B and T cells. However, multiple defects in the clearance of apoptotic cell debris have been found in some lupus patients, which lead to a large amount of uncleared nuclear material that is available for antigen processing and presentation by autoreactive immune cells (Pang, 2014; Li, 2015; Jung 2015). Thus, targeting some of these immune cells for destruction or preventing their activation could be an attractive therapeutic target for treating lupus.

Lupus can affect people of all ages, but is primarily a disease of young women. Approximately 85% of adults with lupus are under age 50, and 28% are under 29 (Feldman, 2013). Although the exact statistics vary by study, non-Caucasian women are at a higher risk of developing lupus than Caucasians. Asian women are twice as likely to develop lupus as Caucasian women, and Hispanic and Native American women are also at increased risk (Johnson, 1995; Samanta, 1991; Feldman, 2013). However, most

dramatically, African American women are 2.5-3.5 times more likely to develop lupus than Caucasian women (Lim, 2014; Chakravarty, 2007; Anderson, 2008). Unfortunately, most cohort studies of lupus patients are made up predominately of Caucasian patients, leaving African Americans and other minorities underrepresented.

The diagnostic criteria for lupus include a broad range of symptoms, and each patient may experience a different set of symptoms. As such, there are many causes of morbidity in lupus patients which can range from mild to life-threatening. In addition to impacting the risk of developing lupus, the patient's ethnicity influences the symptoms that they will experience (Samanta, 1991; Feldman, 2013; Gomez-Puerta, 2015). Many differences can be observed in the incidence of ACR criteria in lupus patients of different ethnicities (Table 1.1). One important difference is that patients of both African American and African Caribbean ethnicity have a significantly higher incidence of renal disorder than Caucasian patients (Somers, 2014; Flower, 2012). It is estimated that lupus nephritis occurs in 55-69% of Hispanic and African American patients and 23-29% of Caucasian patients; nephritis is a major concern, because patients with nephritis have a lower rate of survival than those that do not develop nephritis (Alarcon, 2002; Cervera, 2003; Bastian, 2002). Renal disease is especially threatening for recently diagnosed patients. Patients that have measurable kidney damage soon after diagnosis are more likely to develop end stage renal failure and have a significantly increased mortality rate (Rahman, 2001; Nossent, 2007).

Lupus nephritis is caused by the deposition of immune complexes in the glomerular basement membrane. This begins a process of renal chemokine expression, immune cell infiltration, pro-inflammatory cytokine secretion, tissue damage, and loss of glomerular filtration capacity. Histologically, lupus nephritis can have a wide range of manifestations including: hypercellularity, immune complex deposits, glomerular

Table 1.1 **Clinical manifestations of lupus in different ethnic populations**

Clinical Manifestation	% African American	% African Caribbean	% Caucasian
Malar rash	42.7 †	36.4 *	58.9
Discoid rash	32.2 †	33.1 *	13.5
Photosensitivity	41.4 †	5.8 ‡*	58.9
Oral ulcers	37.9 †	20.9 ‡*	48.2
Arthritis	71.8	84.0 ‡*	69.5
Serositis	45.9	50.3 *	42.3
Renal disorder	40.5 †	47.0 *	18.8
Neurologic disorder	21.7 †	17.2	14.0
Hematologic disorder	66.8	74.1 ‡*	64.5
Immunologic disorder	71.0 †	63.2 ‡	61.3
Antinuclear antibody	96.1 †	95.0 *	91.5

†=significant difference between African American and Caucasian
‡= significant difference between African American and African Caribbean
*= significant difference between African Caribbean and Caucasian

Data presented is compiled from Flower, 2012 and Somers, 2014

sclerosis, crescent formation, and membranous neuropathy. Renal biopsy is used to diagnose nephritis and the International Society of Nephrology/Renal Pathology Society guidelines are used to classify the degree of lupus nephritis depending on the cell types that are affected, and the extent of nephritis in the kidney (Weening, 2004). The immune complexes which are deposited in tissues contain both antibody and antigen. Antibodies reactive to dsDNA, chromatin, and proteins, including a variety of nuclear proteins are found in immune complexes (Mannik, 2003). In healthy individuals, blood-borne immune complexes are rapidly cleared in the liver, a process which is mediated by both complement and Fc receptors; both of these clearance mechanisms are less effective in lupus patients (Edberg, 1987; Davies, 1992; Davies, 2002). Additionally, immune complexes can also be formed *in situ* in the kidney via antibody crossreactivity. Monoclonal anti-dsDNA antibodies and antibodies isolated from the kidneys and urine of lupus patients bind components of the glomerular basement membrane including proteoglycan, heparin sulfate, and hyaluronic acid (Sasaki, 1991; Ben-Yehuda, 1995). Although immune complex formation occurs in all individuals, faulty clearance, an increased amount of cross-reactive anti-nuclear antibodies, and increased availability of nuclear antigen make lupus patients susceptible to immune complex-mediated nephritis. Although renal disease is a significant risk factor for lupus-related mortality, it is not the only cause of lupus-related death.

A recent meta-analysis confirmed that lupus patients are at significantly increased risk of dying compared to the general population, specifically due to increased risk of death from nephritis and infection (Lee, 2016). Encouragingly, the risk for lupus patients of dying from these causes has been steadily decreasing since the 1970s, as has the overall risk of death (Bernatsky, 2006). In the past, it was reported that lupus patients have a high risk of mortality in a bimodal manner, with a large proportion of patients

dying within a year of diagnosis (Urowitz, 1976). However, in recent decades diagnosis and therapies have improved, and lupus patients are not at increased risk of dying soon after diagnosis; in fact, the risk of death is steady over time (Nossent, 2007). Infections and cardiovascular events, including myocardial infarction and heart failure, are the main causes of death among lupus patients, and SLE activity is a common contributing factor (Nossent, 2007). In addition to early kidney damage, a major risk factor for mortality is youth– 40 years old or younger, with those under 24 years old having an even greater risk of mortality (Bernatsky, 2006). Having multiple comorbidities also significantly increases the risk of death for lupus patients. The 1-year mortality rate increases significantly with each comorbidity, from 9% for those with no comorbidities to 30% for those with 2, and 57% for those with over 5 comorbidities (Anderson, 2008). Additionally, ethnicity appears to influence the risk of mortality in lupus patients. African Americans die at a significantly younger age than Caucasians, perhaps in part because they tend to have more comorbidities, including diabetes mellitus, pericarditis, and thrombocytopenia, in addition to nephritis/renal failure (Anderson, 2008). Although the rate of mortality for lupus patients has declined over the past several decades, there is still a need for improved therapeutic options for lupus patients.

As lupus is a chronic disease that affects many women during the reproductive years, the effects of pregnancy on disease activity, and the effects of lupus on pregnancy outcomes, are important considerations. Pregnant lupus patients are at increased risk of maternal and fetal complications, including lupus flare, spontaneous abortion, and preterm birth. The mean gestational age at birth for mothers with lupus is 32 weeks (Feld, 2015). The premature birth rate is 39%, and 23% of pregnancies do not result in live birth (Smyth, 2010). Several factors correlate with adverse fetal and maternal outcomes, especially disease flare within 6 months of conception or during pregnancy,

and prior history of nephritis (Kwok, 2011; Chen, 2015). The risk of fetal loss is significantly decreased in individuals whose lupus is stable over the course of pregnancy. Of women without significant lupus activity, 80% of pregnancies resulted in live births, and 76% ended at full term; among women with significant lupus activity 6 months before pregnancy, only 31% of pregnancies resulted in live birth, and only 23% were delivered at full term (Chen, 2015). Other studies have reported less dramatic fetal mortality, with 89% of asymptomatic women and 65% of women with active lupus achieving live births (Yang, 2014). Regardless of the exact numbers, lupus flare is a significant risk factor for unsuccessful pregnancy outcome. Patients with a prior history of nephritis have a significantly increased risk of flare and associated adverse fetal effects like stillbirth, total fetal loss, fetuses which are small for gestational age, and preterm delivery (Kwok, 2011). To a degree, the risks involved in pregnancy for lupus patients can be managed with careful planning of pregnancies.

Since lupus primarily affects women, especially women between menarche and menopause when estrogen levels are naturally high, it has long been assumed that estrogen plays an important role in the pathogenesis of lupus. This is supported by the fact that exposure to endogenous or exogenous estrogens is a risk factor for developing lupus. Early age of menarche, oral contraceptive use, and post-menopausal HRT increase the risk of developing lupus (Costenbader, 2007). Although men make up only a small proportion of lupus patients, there is evidence that estrogen may contribute to the development of lupus in men. Men with Klinefelter's syndrome (XXY) have levels of serum E2 about twice as high as healthy men, and are at greater risk for developing lupus compared to healthy men (Wang, 1975; Socfield, 2008; Seminog, 2014).

Although estrogen is a risk factor for developing lupus, studies on the effects of estrogen on disease activity in patients with lupus have not been so clear cut. Studies examining

oral contraceptive use and lupus flare have shown mixed results (Jungers, 1982; Sanchez-Guerrero, 2005; Petri, 2005). Similarly, there is no consensus on whether post-menopausal hormone replacement therapy increases the risk of lupus flare (Fernandez, 2006; Buyon, 2005). Over the course of pregnancy, estrogen and prolactin levels increase dramatically, but the incidence of lupus flare correlates more closely with the level of prolactin than with estrogen (Tsesis, 2013; Jara, 2007). Overall, the evidence for estrogen as a risk factor for lupus flare is inconclusive.

There are several ways that estrogens can promote the development of autoimmunity. Besides its impact on B cell development, estrogen can predispose B cells to autoimmunity by impacting the negative selection of autoreactive cells and B cell receptor (BCR) specificity. There are several checkpoints in B cell development where autoreactive B cells are normally removed by induction of apoptosis or anergy; however, estrogen signaling through ERα increases the frequency of high-affinity autoreactive B cells by allowing them to escape negative selection at immature and T2 stages of development, thus allowing the accumulation of autoreactive B cells (Grimaldi, 2006; Hill, 2011). Estrogen promotes the development of autoreactive B cells by increasing the expression of *Bcl-2*, *CD22*, *Shp-1*, and *Vcam-1*, which blunts the BCR signal, and protects against BCR-mediated apoptosis (Grimaldi, 2002). Increased expression of *Bcl-2* alone leads to the production of autoantibodies, and to systemic autoimmunity (Strasser, 1991).

In addition to allowing the survival of autoreactive B cells during development, estrogen also promotes autoimmunity by impacting the specificity of the BCR. Genomic estrogen signaling through EREs leads to expression of activation-induced deaminase which promotes somatic hypermutation and class switch recombination (Mai, 2010; Pauklin, 2009). Somatic hypermutation can lead to *de novo* autoreactivity in B cells that originally

expressed a non-autoreactive BCR (Guo, 2010). Additionally, somatic hypermutation of the BCR promotes T cell activation and the formation of memory T cells (Jiang, 2012). Estrogen also causes an increase in the splenic marginal zone B cell population, a population which is more likely to be autoreactive against some antigens (Hendricks, 2011; Enghard, 2011). Therefore, estrogen affects B cells in multiple ways which promote autoimmunity and immune activation.

As previously discussed, estrogens can signal through two nuclear hormone receptors: ERα and ERβ. Studies of lupus-prone mice have concluded that ERα promotes lupus in these models, while ERβ does not promote disease (Li, 2007; Svenson, 2008; Bynote, 2008). ERα signaling increases the number of Ig-secreting B cells and total serum IgG (Erlandsson, 2003; Li, 2007). In the (NZB x NZW)F1 lupus model, ERα signaling promotes the production of anti-dsDNA IgG autoreactive antibodies, particularly the IgG2a and IgG2b isotypes (Bynote, 2008; Li, 2007).

Exposure to estrogen is a risk factor for developing lupus, yet lupus is still relatively rare among those exposed to estrogen. Aside from other genetic differences which may cause increased susceptibility to lupus, ER-intrinsic factors could impact ER signaling in those prone to lupus, including receptor expression and genetic polymorphisms. The promoter region of *ERα* is significantly demethylated in peripheral blood lymphocytes from lupus patients, which correlates with increased amounts of ERα mRNA and protein in these cells (Liu, 2014; Inui, 2007). It is not known if demethylation of *ERα* occurs before or after the onset of clinical disease. Other studies have reported that lupus patients and healthy controls express the same amount of ERα in B cells, T cells, and monocytes (Suenaga, 1998; Rider, 2006). Despite the proposed differences in *ERα* expression, there are no differences in the binding efficiency and binding characteristics of ERα in lupus patients and healthy controls (Suenaga, 1996).

Polymorphisms in *ERα* are associated with an increased risk of lupus in some populations. The best studied, rs2234693 (commonly referred to as *Pvu*II) and rs9340799 (commonly referred to as *Xba*I), are located in an intronic region between exon 1 and exon 2 of *ERα*. In Caucasian American patients, *Pvu*II and *Xba*I polymorphisms are significantly associated with lupus, and individuals homozygous for either *PvuII* or *XbaI* have an even greater risk of lupus than those with only one copy (Wang, 2010). However in adult Chinese, Korean, and Swedish lupus patients, there is no association of the *Pvu*II and *Xba*I polymorphisms with risk of lupus (Lu, 2009; Lee, 2004; Johansson, 2005). Although the Swedish study did not find an association between these polymorphisms and increased risk of lupus, this study did find that the *PvuII* polymorphism was associated with malar rash and that the *XbaI* polymorphism was associated with photosensitivity (Johansson, 2005). Different combinations of *Pvu*II and *Xba*I alleles are associated with increased production of Th2 cytokines IL-4 and IL-10 in lupus patients (Lu, 2009). Heterozygosity for an exon 8 polymorphism of *ERα,* rs2228480, is also associated with an approximately 3 fold increase in lupus risk (Kassi, 2005). Although these do not by any means represent all of the possible genetic contributions, any of these polymorphisms could predispose individuals to develop lupus by altering their sensitivity to estrogen.

Lupus Treatments

Because lupus is such a heterogeneous disease, there are a range of treatments used, which can be customized to each patient depending on their level of disease activity and symptoms. Most of the current treatments for lupus cause broad immunosuppression which makes patients susceptible to infection. In addition to immunosuppression, many of the drugs used to treat lupus have serious side effects, and these treatments should be given for the shortest amount of time and at the lowest effective dose to avoid side

effects. This can be a problem for a chronic disease like lupus where patients are never cured and may require treatment for the rest of their lives. Current therapies for lupus include: non-steroidal anti-inflammatory drugs (NSAIDs), antimalarials, glucocorticoids, immunosuppressive drugs, and the recently approved antibody belumimab.

NSAIDs, such as ibuprofen and aspirin, are used to treat some mild to moderate symptoms of lupus, including joint pain, mild inflammation, and fever. While these medications have relatively mild side effects, they may not be appropriate for patients with kidney disease because with long-term use they can cause reduced glomerular filtration and increased blood pressure.

Antimalarials hydroxychloroquine and chloroquine are some other commonly used lupus treatments. Use of these medications has increased over the past decade, and about half of lupus patients take antimalarials at some time after diagnosis (Norgaard, 2015). Antimalarials protect against the development of lupus nephritis and end-stage renal disease, and improve the survival of lupus nephritis patients (Galindo-Izquierdo, 2016; Zheng, 2012). Lupus patients taking hydroxychloroquine have a lower risk of serious infection than patients taking immunosuppressive drugs, glucocorticoids, and even those not taking medication (Feldman, 2015). Antimalarials can be used for long term treatment and may be used in combination with other lupus medications. Often, the addition of an antimalarial medication allows patients to use a lower dose of other, more toxic, medications. Antimalarial drugs work via several mechanisms, including interfering with the antigen-presentation function of T cells, antagonism of nucleic acid-sensing TLRs, and by inhibiting the production of pro-inflammatory cytokines (Wallace, 2012; Silva, 2013). Although generally safe, damage to the retina can be a side effect of these drugs.

When NSAIDs and antimalarials cannot effectively control lupus symptoms, glucocorticoids such as prednisone are often prescribed. Glucocorticoids act by binding the glucocorticoid receptor and mimicking the anti-inflammatory effects of cortisol. Glucocorticoids significantly improve the survival of lupus patients (Zheng, 2012). However, there are many side effects of these drugs which can significantly affect the patient's quality of life. Lupus patients that take glucocorticoids have a significantly increased risk of serious infection, which can be life-threatening (Feldman, 2015). Other symptoms include changes in appearance such as weight gain, round face shape, redistribution of fat to the abdomen with thin arms and legs, and fluid retention. Patients taking glucocorticoids can also experience osteoporosis and bone necrosis which can necessitate joint replacement, cataracts, glaucoma, and psychological problems. To decrease the risk of these side effects, glucocorticoids should be taken at the lowest effective dose.

For severe forms of lupus, there are many immunosuppressive drugs that can be used including: azathioprine, cyclosporine, mycophenolate mofetil, methotrexate, leflunomide, and cyclophosphamide. These drugs were originally developed as chemotherapeutic agents or to prevent rejection after an organ transplant and are used for severe lupus that affects organs or organ systems. These drugs interfere with cell replication, suppressing the immune response through various mechanisms, some of which specifically affect immune cells and some of which affect all replicating cells. Many of these medications are "steroid sparing", which means that while taking these drugs, patients are able to take a reduced dose of glucocorticoids. Because these drugs inhibit cell proliferation and the natural immune response, lupus patients taking immunosuppressive drugs are at increased risk of serious infection (Feldman, 2015). An

increased risk of developing cancer later in life is also a side effect of many of these drugs.

In March 2011, the U. S. Food and Drug Administration (FDA) approved belimumab, the first new lupus therapy to be approved in over 50 years. Belimumab was approved for lupus patients without active kidney or central nervous system disease. Belimumab is a human monoclonal antibody against B-cell activating factor (BAFF). BAFF is a B cell growth factor essential for B cell survival. Without BAFF, B cells undergo apoptosis and very few mature B cells develop (Marino, 2014). Overexpression of BAFF leads to both an expanded B cell population and the production of an increased proportion of high-affinity autoreactive B cells (Ota, 2010). BAFF is overexpressed in peripheral blood of lupus patients, and is positively correlated with increased disease activity (Ju, 2006; Duan, 2016). Lupus patients treated with belimumab (in addition to standard of care) have fewer peripheral B cells, including transitional and naïve B cells, pre-switched memory B cells, and plasmablasts/ plasma cells (Jacobi, 2010). They also have significantly less total IgG and less anti-dsDNA antibodies (Stohl, 2012).

Belimumab shows that biologics have great potential to improve on current lupus treatments; however, belimumab itself is far from a magic bullet. After one year of treatment, only 7.5-11.2% more patients treated with belimumab had disease improvement compared to placebo-treated patients (Furie, 2011). Additionally, the risk of sever flare was only reduced from 29.6% in placebo treated patients to 19-20.4% in belimumab treated patients (Stohl, 2012). The cost of belimumab is approximately $35,000 per year, which is quite high for the small percentage of patients who respond positively to this treatment (Lamore, 2012). Furthermore, patients treated with belimumab are also at increased risk of infection.

Another monoclonal antibody, rituximab, is occasionally used to treat lupus, although it is not FDA approved for this purpose. Rituximab is an antibody against CD20, which is present on B cells from the pre-B cell stage in the bone marrow through the memory B cell stage. It is approved for the treatment of B cell leukemia and lymphoma as well as rheumatoid arthritis. Although stage III clinical trials of lupus patients treated with rituximab did not show an improvement over treatment with placebo, it may still be beneficial to some lupus patients, particularly African Americans (Merrill, 2010).

With the exception of NSAIDs taken for minor to moderate inflammation and pain, therapies for lupus are broadly immunosuppressive and can have severe side effects, especially when taken for a long time, as is necessary for a chronic disease like lupus. Overall, the adherence to lupus medications is quite poor. Nonadherence (taking medication appropriately <80% of the time) to lupus medications (prednisone, hydroxychloroquine, other immunosuppressive drugs) has been estimated to be around 50% by multiple studies (Koneru, 2008; Abdul-Sattar, 2015; Ting, 2012). Some of the risk factors for nonadherence include a low education level, not understanding the directions given by the physician, taking more than one medication per day, and taking medications more than once per day (Koneru et al, 2008; Abdul-Sattar 2015). This means that not only can lupus treatment have severe side effects; treatment is also often complicated and burdensome for patients. Physicians that treat lupus patients must try to balance treating the symptoms of lupus, which can themselves be deadly, with the side effects and mental burden caused by treatments. There is an obvious need to improve the treatments for lupus, both in terms of decreasing serious side effects and simplifying the treatment schedule, which should lead to increased patient compliance to medication use.

Research investigating the pathogenesis of lupus has revealed some new potential approaches for the treatment of lupus. Genetic deletion of the *ERα* gene in lupus-prone mice significantly attenuates disease, as does removing the endogenous source of estrogen in young mice by ovariectomy (Bynote, 2008; Sobel, 2005). Among lupus patients, postmenopausal women have fewer flares and a less disease activity compared to premenopausal women (Urowitz, 2006). These data suggest that antiestrogen therapies may be beneficial for lupus patients. Because lupus is predominately a disease of young women, systemic antiestrogens would be inappropriate as they interfere with fertility, and with bone and cardiovascular health. Several different estrogen-modulating drugs are currently used to treat other diseases, but none have been used to treat lupus.

Currently, estrogen-modulating therapies are used to treat and prevent conditions in women including ER-positive breast cancers and osteoporosis. These therapies work by several different mechanisms, from selectively antagonizing ERs to inhibiting the production of estrogen. Selective ER modifiers (SERMs) act as ER agonists in some tissues and as ER antagonists in others. Tamoxifen is a SERM which acts as an ER antagonist in the breast and an ER antagonist elsewhere in the body, and is used for the treatment of ER-positive breast cancers and the prevention of breast cancer in women with a high risk of breast cancer. The SERM raloxifene acts as an ER agonist in bone and an antagonist in the breast and uterus, and is approved for treatment of osteoporosis and to reduce the risk of developing invasive breast cancer in postmenopausal women. Bazedoxifene is a SERM used in conjunction with conjugated estrogens, and is known as a Tissue Selective Estrogen Complex (TSEC). Bazedoxifene is used to prevent osteoporosis and treat moderate-to-severe hot flashes in menopausal and post-menopausal women. Fulvestrant is a pure ER antagonist. It binds ERs, blocks

the binding of endogenous estrogens, and targets ERs for degradation. Aromatase inhibitors are used to block the aromatization of testosterone to estrogen, but these drugs are only effective in postmenopausal women because premenopausal women produce too much aromatase to be effectively inhibited. To inhibit estrogen production in premenopausal women, gonadotropin-releasing hormone agonists can be used; however, these drugs are not appropriate for long-term therapy. Estrogen production can also be permanently stopped by oophorectomy, which causes loss of fertility and premature menopause.

Some of these estrogen-modulating therapies can have unintended effects on tissues throughout the body, and have side effects which may be acceptable for cancer patients who only require treatment for a limited period of time, but are unacceptable for lupus patients who require long-term treatment. The SERMs and TSEC discussed here have been approved by the FDA, and several other SERMs are currently being evaluated. The use of a SERM or targeted delivery of the antiestrogen fulvestrant could be used to antagonize ERs in the cells where ER signaling promotes lupus.

The specific cell type or types that mediate the effects of estrogens on lupus have not been previously identified. We believe that cells of the immune lineage, particularly B cells, promote lupus through ERα signaling. B cells are essential to the development of lupus. Lupus-prone mice without mature B cells do not develop disease (Shlomchik, 1994). Estrogens have many effects of B cells which promote the development of autoimmunity, which are discussed in more detail above. Estrogen signaling through ERα allows autoreactive B cells to escape negative selection at tolerance checkpoints by upregulating anti-apoptotic molecules, promotes affinity maturation of autoreactive B cells, and promotes B cell activation (Grimaldi, 2002; Grimaldi, 2006; Hill, 2011; Guo,

2010; Yoachim, 2015). Depletion of ERα from B cells would mitigate these effects, while avoiding the broad immunosuppression caused by other lupus therapies.

Murine Models of Lupus

There are many murine models used to study lupus. In some models, the onset of lupus can be induced by a single injection with pristane or allogenic donor cells (to induce graft-versus-host disease). One advantage to using an inducible lupus model is that the onset of lupus is uniform among all mice in the study. Drug-induced lupus also occurs in humans after prolonged exposure to certain medications. However, unlike inducible murine lupus, human patients fully recover after discontinuing the medication causing the reaction.

In addition to inducible murine models of lupus, there are other murine lupus models in which disease develops spontaneously as the result of genetic predisposition. Lupus as a result of genetic predisposition is more relevant to human SLE, which has a strong genetic component. Some genetic models of lupus have been used to identify genes that confer lupus susceptibility.

Since lupus is a heterogeneous disease with many different manifestations and symptoms, and no simple genetic cause, there is no perfect murine lupus model. Each model recapitulates some aspects of disease, but none capture the full picture of lupus. Some common genetic models of lupus are the MRL/lpr, BXSB, (NZB x NZW)F1, and NZM2410 mice. These mice all produce antibodies against nuclear antigens which form immune complexes, and glomerulonephritis is the main cause of death in all of these lupus-prone mice. MRL/lpr mice have a spontaneous lymphoproliferation mutation in the Fas gene, which is a receptor essential for Fas/Fas ligand-induced cell death. Mice of both sexes develop systemic autoimmunity, aberrant T cell division, and immune

complex-mediated glomerulonephrosis at a similar rate. In the BXSB strain, male mice develop lupus at a much younger age than females because of the Yaa mutation carried on the Y chromosome. (NZB x NZW)F1 mice are produced by crossing a New Zealand Black (NZB) female with a New Zealand White (NZW) male. The parental NZW strain is healthy and does not develop any lupus-like symptoms. However, NZB mice are prone to leukemia, similar to chronic lymphocytic leukemia, by one year of age (Phillips, 1992). The F1 offspring of this cross inherit risk alleles from each parent, which together confer susceptibility to develop a lupus-like disease. (NZB x NZW)F1 mice of both sexes develop autoantibodies and fatal glomerulonephritis, but females develop these symptoms and die several months before males. The NZM2410 strain was derived from offspring from a female (NZB x NZW)F1 bred with her NZW father (Rudofsky, 1999). Female and male NZM2410 mice develop early-onset severe nephritis. These mice have been extensively used to identify lupus susceptibility loci from the NZB and NZW genomes.

Our lab uses (NZB x NZW)F1 mice for lupus studies. This model recapitulates some of the important aspects of lupus, including the production of antibodies against nuclear antigens, including chromatin and dsDNA, and the development of fatal glomerulonephritis. However, importantly, the (NZB x NZW)F1 model is the only one with a female sex bias. Studies from our lab showed a 100% penetrance of severe glomerulonephritis in both female and male (NZB x NZW)F1 mice by 18 months of age; however, female mice had a median survival time of 238 days while the median survival time of males was 321 days (Bynote, 2008).

Although our lab uses (NZB x NZW)F1s, several different murine lupus models have been used to study the effects of estrogen on lupus. In (NZB x NZW)F1, NZM2410, and MRL/lpr mice, the detrimental effects of estrogen on lupus are mediated by ERα and not

ERβ (Bynote, 2008; Li, 2007; Svenson, 2008; Cunningham, 2014). In lupus-prone mice of both sexes, ERα signaling promotes the production of pathogenic autoantibodies and the production of pro-inflammatory IFN-γ (Bynote 2008; Li, 2007). The activation of B and T cells in (NZB x NZW)F1 mice is also promoted by ERα (Gould Lab, unpublished data). Lupus-prone mice with *ERα* knockout have significant attenuation of albuminuria, decreased lupus-associated kidney damage, and dramatically improved survival (Bynote 2008; Svenson et al, 2008).

Because we know how the lupus phenotype is affected by ERα signaling in lupus-prone (NZB x NZW)F1 mice, we can use this model to identify the cell type or types that cause the negative effects of estrogen on lupus. With this knowledge, we can advance the field and begin to develop targeted antiestrogen therapies for lupus.

Summary

Lupus is a chronic autoimmune disease that predominately affects young women. Exposure to estrogen, both endogenous and exogenous, is a risk factor for developing lupus, and estrogens promote the development of autoimmunity in several ways. Multiple murine lupus models have shown that estrogen promotes lupus via ERα signaling. Taken together, the data presented here support the hypothesis that estrogen signaling through ERα in cells of the immune system, particularly in B cells, promotes lupus. To address this hypothesis, we have performed experiments investigating the role of ERα in both all hematopoietic cells, and B cells in particular, in a lupus-prone mouse model.

The role of ERα in hematopoietic cells was examined using lupus-prone (NZB x NZW)F1 chimeric mice with hematopoietic cells of one *ERα* genotype and non-hematopoietic cells of a different *ERα* genotype. These studies revealed an important role for ERα in

the successful engraftment of hematopoietic cells in female mice, but unfortunately were not able to demonstrate that ERα in hematopoietic cells promotes lupus. To address the role of ERα in B cells on lupus, we produced (NZB x NZW)F1 mice with cre-mediated deletion of *ERα* specifically in B cells. Although we found that the cre knockin allele had its own significant effects and caused relatively low efficiency of *ERα* deletion, we showed that ERα signaling in B cells contributes significantly to the development of lupus. Therefore, our studies have shown that disruption of ERα signaling in B cells is a novel therapeutic target for lupus.

Chapter 2: Dissertation Overview

Hypothesis and Objectives

The objective for these specific aims is to determine the effects of ERα on hematopoietic cells in general, and B cells in particular, on lupus in (NZB x NZW)F1 lupus-prone mice. In specific aim 1 we *hypothesized* that ERα signaling in hematopoietic cells promotes the development of autoimmunity in (NZB x NZW)F1 mice, and that the lack of ERα in hematopoietic cells would attenuate disease. In specific aim 2, we *hypothesized* that ERα signaling in B cells promotes the production of autoantibodies and accelerates mortality in (NZB x NZW)F1 mice.

Rationale

Lupus is a chronic autoimmune disease that predominately affects young women. Exposure to estrogen, both endogenous and exogenous, is a risk factor for developing lupus, and estrogens promote the development of autoimmunity by impacting the development and activation of immune cells. Multiple murine lupus models have shown that estrogen promotes lupus via ERα signaling. In these studies, ERα signaling was affected throughout the body, and the specific cell type or types which promote lupus via ERα were not elucidated. Therefore, our lab sought to determine the cell type or types which mediate the effects of ERα on lupus.

In lupus patients, several types of immune cells function abnormally in ways which contribute to lupus. Impaired negative selection leads to the persistence of autoreactive B cells and the development of autoreactive plasma cells. Macrophages from lupus patients have an impaired ability to phagocytose apoptotic debris, leading to an increased presence of nuclear antigens. Follicular dendritic cells present autoantigens from apoptotic cell debris to B cells, resulting in the activation of autoreactive B cells. Some of these abnormalities have been shown to be dependent on ERα. *ERα* is

expressed by hematopoietic cells at different stages and in different lineages including: HSCs, MPPs, common myeloid progenitors, common lymphoid progenitors, B cells, T cells, and myeloid cells (Sanchez-Aguilera, 2014).

B cells are essential to the development of lupus. Lupus-prone mice without B cells have a completely attenuated lupus phenotype (Shlomchik, 1994). Estrogens have many effects on B cells which promote the development of autoimmunity. Estrogen signaling through ERα allows autoreactive B cells to escape negative selection at tolerance checkpoints by upregulating anti-apoptotic molecules, and promotes affinity maturation of autoreactive B cells (Grimaldi, 2002; Grimaldi, 2006; Hill, 2011; Guo, 2010). ERα also promotes the activation of B cells in female mice carrying lupus susceptibility loci (Yoachim, 2015).

Additionally, $CD4^+$ T cells are stimulated by E2 through ERα, resulting in a Th-1 immune response and IFN-γ production (Maret, 2003). In lupus-prone mice, ERα promotes T cell activation, and *ERα* knockout causes a significant decrease in the serum level of IFN-γ (Yoachim, 2015; Bynote, 2008). In contrast to its effect on $CD4^+$ T cells, ERα signaling in antigen-presenting splenic macrophages leads to decreased stimulation of $CD4^+$ T cells and a reduced Th-1 response (Lambert, 2005). These data suggest that ERα signaling regulates the immune response by causing different effects in different types of immune cells.

Taken together, these data strongly suggest that ERα signaling in hematopoietic cells plays a role in the development of autoimmunity. Therefore, we were interested to investigate the effects of loss of ERα in hematopoietic cells on lupus. Because B cells play such a central role in lupus, and because of the strong evidence that ERα in B cells promotes the development of autoimmunity, we were also interested in the role that ERα

in B cells plays in lupus. The results of these studies will give new insight into the pathogenesis of lupus, and could reveal novel therapeutic targets for the treatment of lupus.

Specific Aims

Aim1: To determine how ERα signaling in hematopoietic cells impacts lupus in (NZB x NZW)F1 mice.

The role of ERα in hematopoietic cells was examined using lupus-prone (NZB x NZW)F1 chimeric mice transplanted with hematopoietic cells of a different *ERα* genotype. The engraftment of transplanted cells in these chimeric mice was determined by QPCR analysis of peripheral blood DNA. Additionally, the survival of host plasma cells was analyzed by QPCR of isolated plasma cells, and the antigen specificity of these cells was analyzed by ELISPOT. The development of lupus including autoantibody production, glomerulonephritis, and survival was analyzed in these mice. The results of these studies are presented in chapter 3.

Aim 2: To determine how ERα signaling in B cells affects lupus in (NZB x NZW)F1 mice.

To address the role of ERα in B cells on lupus, we produced (NZB x NZW)F1 mice with cre-mediated deletion of *ERα* specifically in B cells using the CD19-Cre knockin allele. The efficiency of cre-mediated deletion of *ERα* was examined using QPCR. Survival was monitored for up to one year. The production of antibodies and autoantibodies was analyzed by ELISA. Histology was used to assess the development of glomerulonephritis, and flow cytometry was used to assess the impact of *ERα* deletion on B cell development. The results of these studies are presented in chapter 4.

Chapter 3: Loss of estrogen receptor alpha in hematopoietic cells temporarily attenuates lupus and impacts hematopoietic reconstitution

Abstract

Estrogen signaling through ERα promotes lupus in lupus-prone (NZB x NZW)F1 mice. Knockout of *ERα* in (NZB x NZW)F1 mice significantly attenuates autoantibody production, glomerulonephritis, and mortality. We hypothesized that ERα signaling in hematopoietic cells was responsible for these effects. To investigate this, we created chimeric (NZB x NZW)F1 mice with different combinations of $ERα^{+/+}$ and $ERα^{-/-}$ hematopoietic and non-hematopoietic cells. $ERα^{-/-}$ hematopoietic and $ERα^{-/-}$ non-hematopoietic cells negatively impacted engraftment in female, but not male, mice. Female $ERα^{+/+}$ mice given $ERα^{-/-}$ hematopoietic cells had a lower rate of engraftment than those given $ERα^{+/+}$ cells, and female $ERα^{-/-}$ mice that received $ERα^{+/+}$ hematopoietic cells had a low level of engraftment, which decreased over time. In mice that were successfully transplanted, the genotype of the transplanted bone marrow had no impact on the latency to development of albuminuria or the development of glomerulonephritis. Furthermore, there was no difference in the survival of $ERα^{+/+}$ mice that were successfully transplanted with either $ERα^{-/-}$ or $ERα^{+/+}$ hematopoietic cells. However, we did find that $ERα^{+/+}$ females that received $ERα^{-/-}$ hematopoietic cells produced fewer autoantibodies at a young age. However, this difference did not persist over time. We postulate that the delayed production of pathogenic autoantibodies in $ERα^{+/+}$ mice that were transplanted with $ERα^{-/-}$ hematopoietic cells was the result of residual host-derived ($ERα^{+/+}$) plasma cells. Up to ten months after transplantation, host-derived plasma cells were detected in some female and male mice. Furthermore, through analysis of irradiated, but untransplanted mice, we showed that although immune cells were significantly depleted, a significant amount of $ERα^{+/+}$ dsDNA-reactive plasma cells

survived irradiation. Altogether, these data suggest that residual autoreactive *ERα*$^{+/+}$ hematopoietic cells may be sufficient to drive lupus in (NZB x NZW)F1 mice. Additionally, these studies demonstrated that ERα signaling modulates the hematopoietic reconstitution potential in female mice.

Background

ERα signaling promotes the pathogenesis of lupus in (NZB x NZW)F1 mice. It causes increased production of autoantibodies, increased glomerulonephritis, and shorter survival (Bynote, 2008; Li, 2007). These effects were observed in models where mice had *ERα* knockout or ERα stimulation throughout the body. Because these models looked at the effects of ERα in the entire body, they cannot be used to identify which cell type or types mediate the pathogenic effects of ERα. For this, other models must be developed where the effects of ERα can be examined in specific cell types.

Hematopoietic stem cells give rise to the cells of the immune system including B cells, T cells, macrophages, and dendritic cells. Lupus patients have many hematologic abnormalities, including a reduced number of peripheral leukocytes. Some of the abnormalities observed in the immune cells of lupus patients may contribute to the development and survival of autoreactive B and T cells and the development of autoimmunity.

Some of the immune alterations found in lupus patients are related to defective clearance of apoptotic cell debris, which can be a source of nuclear autoantigens if not properly degraded. Lupus patients have a higher proportion of apoptotic white blood cells, and are not able to clear apoptotic debris as rapidly as healthy people (Fan, 2014; Gaipl, 2007). Both monocyte-derived and tingible body macrophages from lupus patients are impaired in their ability to phagocytize apoptotic cells, which leads to the accumulation of apoptotic debris in germinal centers (Tas, 2006; Baumann, 2002). This free apoptotic debris can be taken up by follicular dendritic cells and presented to B cells, and lead to activation of autoreactive B cells (Gaipl, 2007).

Defective clearance of apoptotic bodies, and presentation of nuclear antigens by follicular dendritic cells can lead to the activation of autoreactive B cells, but lymphocytes from lupus patients have other characteristics which can also contribute to aberrant activation of the immune system. Anti-inflammatory Bregs from lupus patients have a decreased ability to inhibit T cell proliferation, independent of any lupus-related T cell defects (Gao, 2014). Additionally, T cells from lupus patients have more rapid and robust responses to T cell receptor stimulation (Vassilopoulos, 1995; Liossis, 1998). Both $CD4^+$ and $CD8^+$ T cells substantially contribute to the production of anti-dsDNA IgG by B cells in lupus patients (Linker-Israeli, 1990). Alterations in B and T cell function can lead to synergistic B and T cell activation which, combined with a less effective Breg population, can lead to increased immune activation. This may contribute to the increase in B cells and plasma cells, and the higher percentage of activated B cells found in lupus patients (Fan, 2014; Korganow, 2010).

Although these particular effects have not been explicitly linked to estrogen, estrogen affects the immune system in many ways that promote the development of autoimmunity. Estrogen (through ERα) promotes the development of autoreactive B cells by allowing high-affinity autoreactive B cells to escape negative selection at tolerance checkpoints (Grimaldi, 2006; Hill, 2011, Grimaldi, 2002). Estrogen also promotes B cell autoreactivity by promoting somatic hypermutation in the periphery, which can cause mutations that lead to an autoreactive BCR (Mai, 2010; Pauklin, 2009; Guo, 2010). Additionally, somatic hypermutation causes T cell activation and the formation of memory T cells (Jiang, 2012).

In addition to its effects on B cell autoreactivity, estrogen may promote lupus by stimulating Th2 immune responses. E2 promotes the activation of both $CD4^+$ and $CD8^+$ T cells (Wang, 2008). Exposure to estrogen causes $CD4^+$ T cells to produce fewer Th1

and more Th2 cytokines, thus promoting a humoral immune response (Polanczyk, 2005; Haghmorad, 2014). This is protective in the EAE model of multiple sclerosis (Lelu, 2011), where E2 protects against the development of disease, but may be pathogenic in lupus which is mediated by the Th2 immune response. Additionally, E2 prevents Fas-dependent apoptosis in activated Th2, but not Th1, cells by increasing Bcl-2 expression (Huber, 1999). A similar phenomenon is seen in lupus patients where estrogen inhibits the activation-induced apoptosis of T cells by down-regulating the expression of FasL (Kim, 2010).

Not all T cells promote Th2 immune responses in response to estrogen signaling. iNKT cells produce IFN-γ, a Th1 cytokine, in response to estrogen signaling through ERα (Gourdy, 2005). Exposure to estrogens increases the number of T regs and enhances their capacity to suppress $CD4^+$ effector T cells (Polanczyk, 2005). The anti-inflammatory effects of Tregs have been suggested to be dependent on Treg intrinsic expression of *ERα* (McKarns, 2015). Thus, not all of the effects of *ERα* on hematopoietic cells promote immune stimulation and the development of autoimmunity.

Additionally, estrogens promote autoimmunity by acting on myeloid lineage cells. Chronic ERα stimulation by E2 causes increased production of inflammatory cytokines and nitric oxide by macrophages (Calippe, 2008; Calippe, 2010). E2 promotes the differentiation of dendritic cells with potent antigen-presentation capabilities, which strongly promote the proliferation of $CD4^+$ T cells (Paharkova-Vatchkova, 2004). ERα stimulation also causes dendritic cells to produce inflammatory IFN-α and TNF-α (Seillet, 2012). Together, these studies show that ERα promotes autoimmunity though multiple mechanisms by acting on both lymphoid and myeloid cells. Therefore, we hypothesized that cells of the hematopoietic lineage are likely responsible for ERα's role in promoting lupus.

To study the impact of ERα in hematopoietic cells on lupus, we created (NZB x NZW)F1 chimeras by transplanting *ERα*$^{-/-}$ and *ERα*$^{+/+}$ hematopoietic cells into mice with that were *ERα*$^{+/+}$ elsewhere in the body. Because ERα in stromal cells plays an important role in the development of the cells that the stroma supports (as demonstrated in the thymus and testis), we also transplanted *ERα*$^{+/+}$ hematopoietic cells into *ERα*$^{-/-}$ and *ERα*$^{+/-}$ recipients.

Methods

Production of experimental mice

All animals were housed under conditions of controlled humidity, temperature, and lighting in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the *Guide for the Care and Use of Laboratory Animals*. Mice had ad libitum access to 7904 Teklad Irradiated S-2335 Mouse Breeder Diet (Harlan Teklad, Madison, WI, USA). All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Age and sex matched (NZB x NZW)F1 mice between 8-12 weeks old were used as both cell donors and recipients for these studies. Recipient mice were irradiated with two doses of 5 Gy, 4 hours apart with a RS-2000 irradiator in the Biological Irradiator Core (RAD Source Technologies Inc., Alpharetta, GA, USA).

Bone marrow and spleen cells were isolated from donor mice in a laminar flow hood under sterile conditions. Cells were suspended in RPMI cell culture medium (Gibco, Waltham, MA, USA) at $1x10^7$ live cells/ 100 µl. Approximately $2x10^7$ bone marrow cells and $1x10^7$ splenocytes were injected i.v. via the tail vein immediately after the second dose of irradiation. Mice were given antibiotic-supplemented water (1 g/L neomycin and 125 mg/L polymyxin B) 4 days before transplant until 2 weeks post-transplant, to prevent infections while the transplanted immune system was established (Sigma-Aldrich, St. Louis, MO, USA; Calbiochem, San Diego, CA, USA).

The following combinations of transplants were performed. $ERα^{+/+}$ or $ERα^{-/-}$ cells into $ERα^{fl/+}$ recipients, and $ERα^{+/+}$ cells into $ERα^{+/-}$ or $ERα^{-/-}$ recipients, outlined in Table 1.

The floxed (fl) allele of *ERα* is functionally a wild type allele, and (NZB x NZW)F1.ERα$^{+/-}$ mice develop lupus which is indistinguishable from (NZB x NZW)F1.ERα$^{+/+}$ mice.

Analysis of engraftment efficiency

Blood was collected 8 weeks after transplant, and red blood cells lysed with ACK lysis buffer (Gibco). DNA was then isolated with a DNeasy kit using the protocol for cultured cells (Qiagen, Hilden, Germany). Blood DNA was analyzed for engraftment using Sybr green (Applied Biosystems, Foster City, CA, USA) quantitative PCR (QPCR) and a 7500 Real Time PCR System (Applied Biosystems). For ERα$^{-/-}$→ERα$^{fl/+}$ and ERα$^{+/+}$→ERα$^{fl/+}$ transplants, ERαfl F: 5'-CTATACGAAGTTATGGATCCCTAGC-3' and ERαfl R: 5'-CACATGCAGCAGAAGGTATTTG-3' primers were used to detect the ERα^{fl} allele (149 bp product) and ERαEx5 F: 5'-GGAAGGCCGAAATGAAATGGG-3' and ERαEx5 R: 5'-CCAACAAGGCACTGACCATC-3' primers were used to detect exon 5 of ERα (140 bp product). Exon 5 of *ERα* is identical in the *ERα^{fl}*, *ERα^{-}*, and *ERα^{+}* alleles. Grafts with ≥95% efficient engraftment (<5% *ERα^{fl}* DNA detected) were considered successful for these sets of transplants. For ERα$^{+/+}$→ERα$^{-/-}$ and ERα$^{+/+}$→ERα$^{+/-}$ transplanted mice, NeoC F: 5'-GGCATTCTGCACGCTTCAAA-3' and NeoC R: 5'-TTGTTCAATGGCCGATCCCA-3' (104 bp product) primers were used to detect the *ERα^{-}* allele, and compared to ERα exon 5. The NeoC primers over-reported the amount of *ERα^{-}* DNA present in samples by ~5%, so the cutoff for successful engraftment was placed at 90%.

Analysis of chemokines and receptors

Bone marrow was collected from 3 month old (NZB x NZW)F1 *ERα$^{-/-}$* and *ERα$^{+/+}$* mice. RNA was isolated from cells using the Absolutely RNA Miniprep Kit (Aligent Technologies, Santa Clara, CA, USA). Up to 2.5 µg RNA was converted to cDNA using SuperScript VILO Master Mix (Invitrogen, Carlsbad, CA, USA) and was diluted 1:80

before use. RT PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: Cxcr4 F: 5'-AAACCTCTGAGGCGTTTGGT-3' and Cxcr4 R: 5'-GCAGGGTTCCTTGTTGGAGT-3' (148 bp product), Cxcr7 F: 5'-ACAGGCTATGACACGCACTG-3' and Cxcr7 R: 5'-ACGAGACTGACCACCCAGAC-3' (92 bp product), Cxcl12 F: 5'-CAGAGCCAACGTCAAGCA-3' and Cxcl12 R: 5'-AGGTACTCTTGGATCCAC-3' (128 bp product). Each of these primers was normalized to GAPDH. The GAPDH primers were, F: 5'-TGCACCACCAACTGCTTAG-3' and R: 5'-GGATGCAGGGATGATGTTC-3' (177 bp product).

Survival studies

Mice were monitored biweekly for albuminuria with Albustix (Bayer Corporation, Elkhorn, IN, USA). Upon a positive (2+ or greater) reading, mice were tested weekly. Mice were considered positive for albuminuria upon two consecutive readings of 2+ (100 mg/dl) or greater. Mice were also visually examined weekly for physical symptoms of distress. Mice were sacrificed upon two consecutive albuminuria readings of 4+ (>2000 mg/dl) or upon signs of significant distress.

Serological analysis

Enzyme Linked Immunosorbent Assay (ELISA) was used to measure serum antibody levels. Anti-chromatin IgG ELISA plates were made with Immulon 2 HB plates (Thermo Fisher Scientific, Waltham, MA, USA) coated with excess histone proteins and dsDNA. Diluted serum samples were incubated on the plate. The plate was washed, and then incubated with anti-mouse IgG HRP conjugate (EMD Millipore, Billerica, MA, USA). After washing, TMB substrate (Alpha Diagnostic International, San Antonio, TX, USA) was added for 20 minutes or until a significant color change developed, after which Stop Solution (Alpha Diagnostic International) was added to the wells, and the plate was read

on a 96 well microplate reader at 450 nm. For wash steps, 0.05% Tween-20 in PBS was used. Anti-dsDNA IgG ELISAs were done with Immulon 2 HB plates (Thermo Fisher Scientific) coated with only dsDNA, and the same procedure was followed as for the anti-chromatin ELISAs.

Flow cytometry

To assess cells by flow cytometry, bone marrow and spleen cells were isolated from mice at the end of the survival study. Bone marrow cells were gently pipetted up and down and spleen cells were passed through a 70 μm cell strainer (Thermo Fisher Scientific) to create single cell suspensions. Red blood cells were lysed with ACK lysis buffer (Gibco) and stained with combinations of CD138 PE, CD19 APC, CD27 V450, CD4 PE, CD25 APC-Cy7, CD62L APC, CD69 FITC, CD80 FITC, and CD86 PE (all BD Biosciences, San Jose, CA, USA). Propidium iodide was used as a live/dead stain (BD Biosciences). Cells were fixed with 0.6% formalin. Flow cytometry was performed with a BD LSRII Flow Cytometer and analyzed with FACSDiva software (BD Biosciences, v. 8.0).

Periodic acid-Schiff staining and analysis

Kidneys were collected from mice upon death or sacrifice at the end of the survival study and fixed in 10% formalin for 24 hours. Fixed kidneys were paraffin embedded, sectioned, stained with the Periodic Acid-Schiff Kit (Sigma-Aldrich), and analyzed by light microscopy. One hundred glomeruli per kidney were evaluated as previously described (Bynote, 2008). Briefly, kidneys with 20-50% affected glomeruli have moderate glomerulonephritis, and those with < 50% affected glomeruli have severe glomerulonephritis.

Plasma cell isolation and genotyping

Plasma cells were isolated from mice at the time of death or at one year of age. Briefly, single cell suspensions were made from the bone marrow and spleen, and red blood cells were lysed. The $CD138^+$ plasma cell kit and AutoMacs (Miltenyi Biotech, Bergisch-Gladbach, Germany) were used to isolate $CD138^+$ plasma cells. DNA was collected from these cells using the DNeasy kit (Qiagen) and QPCR was performed as described above for engraftment efficiency using ERαfl and ERαEx5 primers.

ELISPOT

To examine the potential of autoreactive plasma cells to survive irradiation, 10 week old mice were irradiated with two doses of 5 Gy four hours apart, but were not injected with cells from a donor mouse. After irradiation, mice were monitored three times daily for signs of distress. 7-8 days after irradiation, mice were sacrificed and bone marrow and spleen cells were collected, single cell suspensions made, and stained for fluorescence activated cell sorting (FACS) with CD138-PE, MHCII-V500, and propidium iodide as a live dead gate (BD Biosciences). FACS was used to purify populations of live long-lived ($CD138^+$ $MHCII^{lo}$) and short-lived ($CD138^+$ $MHCII^{hi}$) plasma cells. Cells were sorted with a FACSAria II (BD Biosciences). Isolated cells in RPMI +10% fetal bovine serum were applied to an Immulon 2 HB plate coated with dsDNA and were incubated at 37ºC with 6% CO_2 in a humid environment for 24 hours. Biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was applied, followed by streptavidin-alkaline phosphatase (Southern Biotech, Birmingham, AL, USA). Next, 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) in AMP buffer was applied until colored spots developed. The plate was rinsed with running water and spots counted under a dissecting microscope.

Statistics

Statistical analysis was done using SPSS software (IBM, v. 23). P values of less than or equal to 0.05 are considered significant. Kaplan Meyer survival curves and log rank tests were used to evaluate survival. A 2-sided Fisher's exact test was used to analyze the engraftment efficiency of ERα^{fl} transplants. Student's t tests were used to evaluate the efficiency of some transplant, ELISA, flow cytometry, histology, and QPCR data.

Results

Production of Experimental Animals

To analyze the effect of ERα in hematopoietic cells on lupus in (NZB x NZW)F1 mice, we produced two sets of chimeric mice. The first set was designed to compare the effects of $ERα^{-/-}$ and $ERα^{+/+}$ hematopoietic cells in $ERα^{+/+}$ (NZB x NZW)F1s. The second set used $ERα^{+/+}$ hematopoietic cells in both $ERα^{-/-}$ and $ERα^{+/-}$ recipients to evaluate the contributions of ERα in non-hematopoietic cells to lupus. The first set of chimeras was produced by transplantation of $ERα^{+/+}$ or $ERα^{-/-}$ bone marrow into lethally irradiated $ERα^{fl/+}$ recipients ($ERα^{+/+}$→$ERα^{fl/+}$ and $ERα^{-/-}$→$ERα^{fl/+}$). The loxP sites in the $ERα^{fl}$ allele are located in introns surrounding *ERα* exon 3, and functions as $ERα^{+}$ allele when it has not undergone recombination (as is the case in this cre recombinase-free system) (Dupont, 2000). Transplantation of hematopoietic cells to $ERα^{fl/+}$ recipients allowed us to detect cells derived from the host mouse in QPCR assays.

The second set of chimeric mice was produced by transplanting $ERα^{+/+}$ bone marrow into lethally irradiated $ERα^{+/-}$ or $ERα^{-/-}$ mice ($ERα^{+/+}$→$ERα^{+/-}$ and $ERα^{+/+}$→$ERα^{-/-}$). For this set of chimeras, cells from the host can be detected by QPCR for the null allele of *ERα*. $ERα^{+/-}$ mice were used as recipients because the null allele of *ERα* could be detected by QPCR to determine engraftment efficiency. Heterozygosity for the null allele of *ERα* has no impact on the lupus phenotype in (NZB x NZW)F1 mice (Bynote, 2008). The chimeric mice used for these studies are named so that the first genotype indicates the donated hematopoietic cells and the second indicates the genotype of the recipient mouse and the non-hematopoietic cells (Table 3.1).

Table 3.1 **The *ERα* genotype of hematopoietic and non-hematopoietic cells in chimeric mice**

Transplant	Hematopoietic cells	Non-hematopoietic cells
$ERα^{-/-} \rightarrow ERα^{fl/+}$	$ERα^{-/-}$	$ERα^{fl/+}$
$ERα^{+/+} \rightarrow ERα^{fl/+}$	$ERα^{+/+}$	$ERα^{fl/+}$
$ERα^{+/+} \rightarrow ERα^{-/-}$	$ERα^{+/+}$	$ERα^{-/-}$
$ERα^{+/+} \rightarrow ERα^{+/-}$	$ERα^{+/+}$	$ERα^{+/-}$

Optimization of the Transplantation Protocol

Before these experiments began, preliminary experiments were carried out to optimize the transplantation protocol. Different strains of mice can tolerate different amounts of irradiation, and when we began these experiments there was no information available about the amount of irradiation (NZB x NZW)F1 mice could tolerate. A protocol from another lab at our institution specified that mice should be irradiated with 10 Gy (1000 rad) prior to transplant to produce chimeric mice (Garg, 2009). When 10 Gy of x-ray irradiation was administered to (NZB x NZW)F1 mice in a single dose, followed immediately by cell transplant, mice lost an average of 15% of their body weight and showed visible signs of distress before they died, an average of 12 days after the procedure. Other available protocols specified that irradiation should be given in a split dose, with a four hour gap between doses. When (NZB x NZW)F1 mice were irradiated with a split dose of irradiation (5 Gy, then four hours later 5 Gy) and then immediately transplanted with hematopoietic cells, the mice did not develop visible signs of distress, and only lost an average of 3% of their initial body weight after 2 weeks. The survival of (NZB x NZW)F1s treated with single and split dose irradiation is shown in Figure 3.1a.

To determine if the irradiation and transplantation procedure had a significant impact on the long-term survival of (NZB x NZW)F1 mice, the survival of a small group of female mice transplanted with *ERα*$^{+/-}$ bone marrow was monitored. These mice had a median survival of 211 days, which is not different from the 239 day median survival time of unirradiated *ERα*$^{+/-}$ females (Figure 3.1b). Thus, this split dose irradiation protocol did not negatively impact the median survival time in (NZB x NZW)F1 mice.

ERα is necessary for Efficient Bone Marrow Engraftment in Females but not Males

The engraftment efficiency of bone marrow transplants was measured with DNA isolated from peripheral blood 8 weeks after transplant. The efficiency of ERα$^{-/-}$→ERα$^{fl/+}$ and

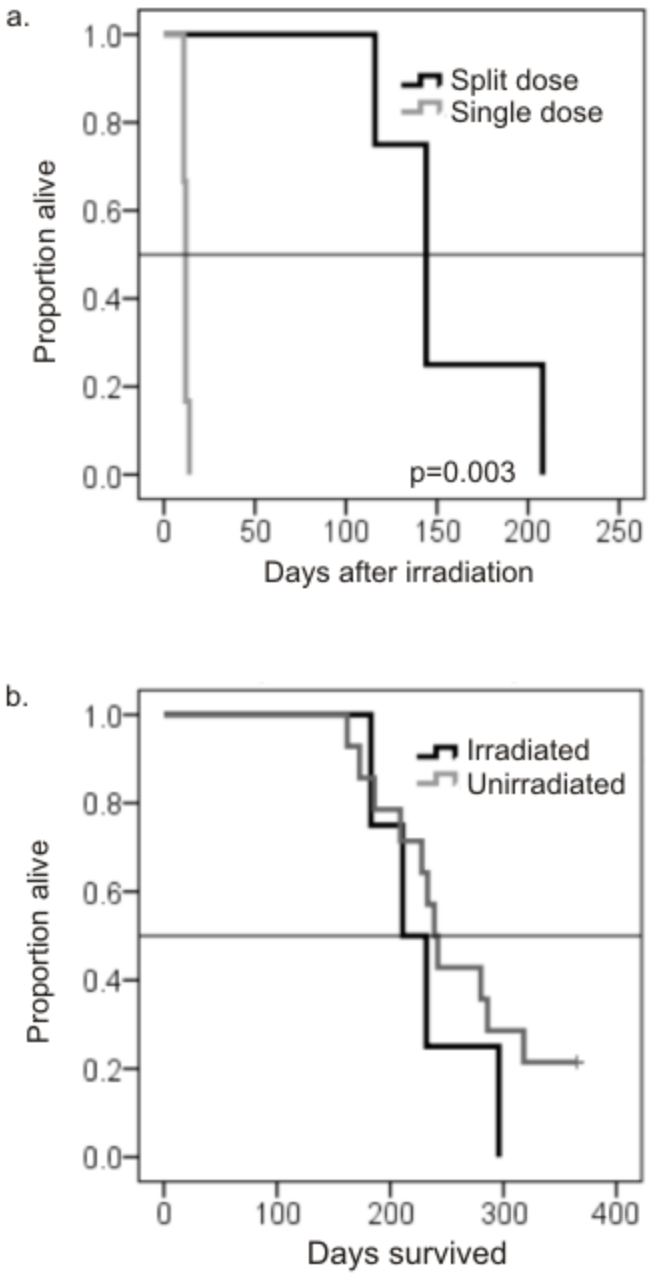

Figure 3.1 **Split dose irradiation does not significantly affect survival of (NZB x NZW)F1 mice**

Figure 3.1 **Split dose irradiation does not significantly affect survival of (NZB x NZW)F1 mice**

(NZB x NZW)F1 mice were irradiated with 10 Gy of x-ray irradiation in single and split doses and transplanted with hematopoietic cells. (a) Mice that received a single dose of irradiation died soon after transplant while mice that received a split dose survived much longer (p=0.003). The median survival (b) is not different between female mice treated with a split dose of irradiation and female mice that were not irradiated.

$ERα^{+/+}→ERα^{fl/+}$ transplants was determined by measuring the relative abundance of recipient-derived DNA (as measured by the $ERα^{fl}$ allele) by QPCR. Efficiency of $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ transplants was determined by measuring the amount of recipient-derived DNA (as measured by the $ERα^{-}$ allele) by QPCR. $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ transplants with ≥ 95% engraftment were considered successful, and unsuccessfully transplanted mice were excluded from further studies. The primers used to measure $ERα^{-}$ DNA slightly over-represent the amount of $ERα^{-}$ DNA in each sample, so in engraftment is slightly overestimated in $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ transplants, so mice with ≥ 90% estimated engraftment were considered successful.

For female mice, there was a statistically significant difference in the transplant efficiency between mice with different *ERα* genotypes in hematopoietic cells. In females, the success rate of the $ERα^{-/-}→ERα^{fl/+}$ grafts was significantly less than that of the $ERα^{+/+}→ERα^{fl/+}$ grafts (Figure 3.2a). Among $ERα^{-/-}→ERα^{fl/+}$ females, only 35% had successful grafts, with a median of 90% engraftment, while in $ERα^{+/+}→ERα^{fl/+}$ females, 68% had successful grafts with a median of 100% engraftment.

There was also a statistically significant difference in the transplant efficiency, measured 8 weeks after transplant, between female mice with different *ERα* genotypes of non-hematopoietic cells. The estimated rate of engraftment in $ERα^{+/+}→ERα^{-/-}$ females was significantly lower than in $ERα^{+/+}→ERα^{+/-}$ females (Figure 3.2b). Among $ERα^{+/+}→ERα^{+/-}$ females the median estimated engraftment was 88.5%, while in $ERα^{+/+}→ERα^{-/-}$ females the median estimated engraftment of was 39%. This indicates that loss of *ERα* in both hematopoietic and non-hematopoietic cells significantly impacts the success of hematopoietic reconstitution in females.

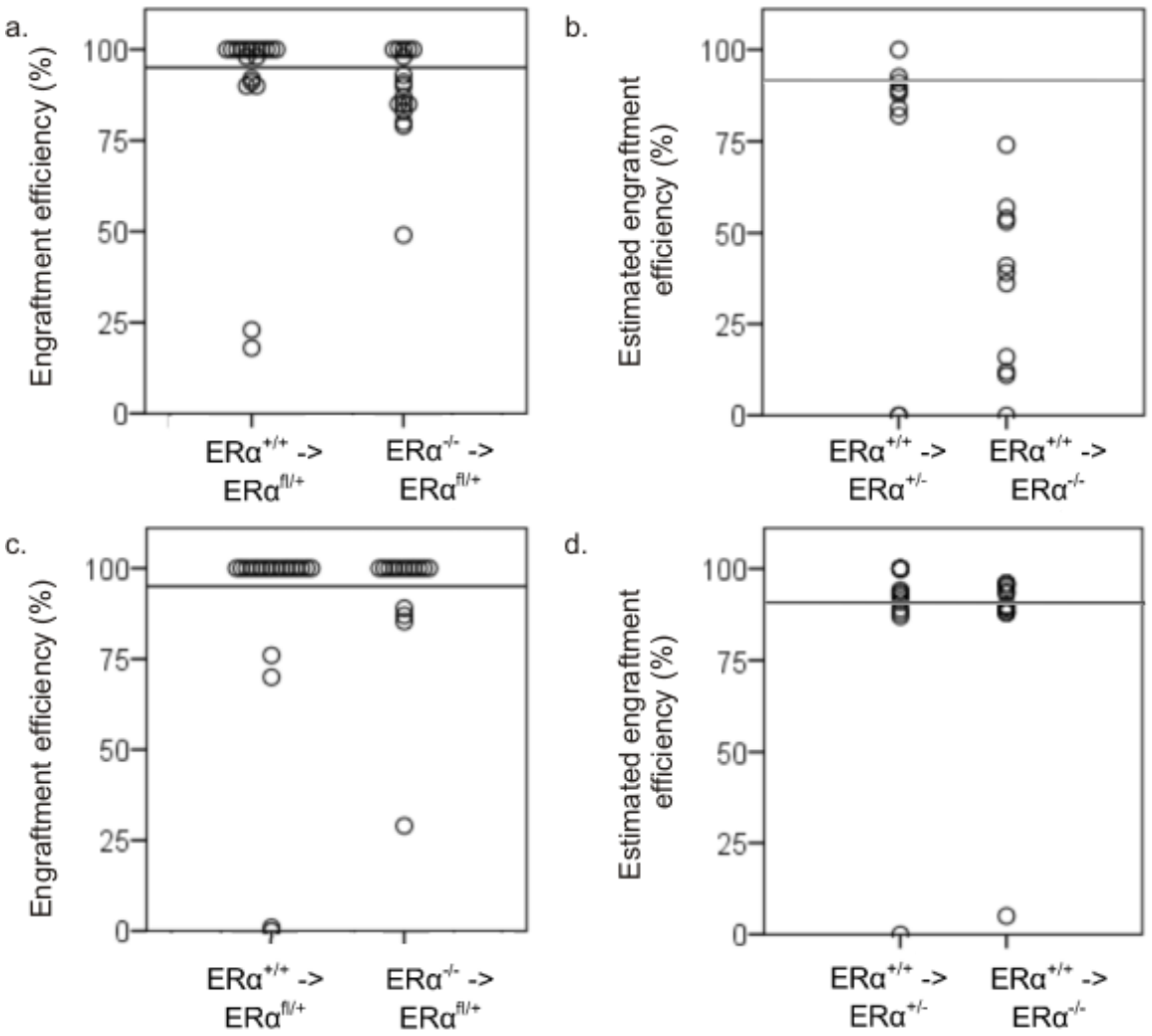

Figure 3.2 **Loss of *ERα* causes impaired hematopoietic reconstitution in female mice**

Figure 3.2 **Loss of *ERα* causes impaired hematopoietic reconstitution in female mice**

Engraftment efficiency was detected by QPCR of DNA from peripheral blood. (a) The group of female $ERα^{-/-}$→$ERα^{fl/+}$ chimeras had significantly fewer successful transplants compared to $ERα^{+/+}$→$ERα^{fl/+}$ females (p=0.047). (b) Female $ERα^{+/+}$→$ERα^{-/-}$ chimeric mice also had significantly lower rates of engraftment compared to $ERα^{+/+}$→$ERα^{+/-}$ females (p=0.016). Among male mice (c-d) the ERα genotype of hematopoietic and non-hematopoietic cells did not affect transplant success.

The engraftment efficiency in male mice did not depend on ERα genotype. There was no difference in the proportion of successful transplants between $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ males (Figure 3.2c). 73% of $ERα^{-/-}→ERα^{fl/+}$ males had successful grafts with a median engraftment of 100%, and 80% of $ERα^{+/+}→ERα^{fl/+}$ males had successful grafts with a median of 100% engraftment. Likewise, $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ male mice did not differ in transplantation efficiency (Figure 3.2d). The median estimated engraftment efficiency was 90% for $ERα^{+/+}→ERα^{-/-}$ males, and 93% for $ERα^{+/+}→ERα^{+/-}$ males. Therefore, loss of *ERα* in either hematopoietic or non-hematopoietic cells does not impact the success of hematopoietic reconstitution in males.

In addition to successful initial engraftment (measured 8 weeks after transplant, at about 4 months of age), successful bone marrow transplants must be able to maintain engraftment over time. To assess engraftment efficiency over time, we isolated DNA from blood collected at monthly intervals, and used QPCR to measure the $ERα^{fl}$ or $ERα^{-}$ DNA originating from the host in selected samples. At least 3 mice of each sex and genotype were evaluated at 6 months of age, and some were additionally evaluated at 8 and 10 months of age.

In both female and male $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ mice, the engraftment efficiency was stable between 4-8 months of age for females and 4-10 months for males (Figures 3.3a-d). The degree of engraftment did not significantly decrease in either set of female mice between 4-6 months, or male $ERα^{-/-}→ERα^{fl/+}$ mice between 4-10 months. The degree of engraftment did significantly decrease between 4 and 10 months in $ERα^{+/+}→ERα^{fl/+}$ males, however this was a small decrease, corresponding to less than 2% per month (average 100% at 4 months vs 93% at 10 months).

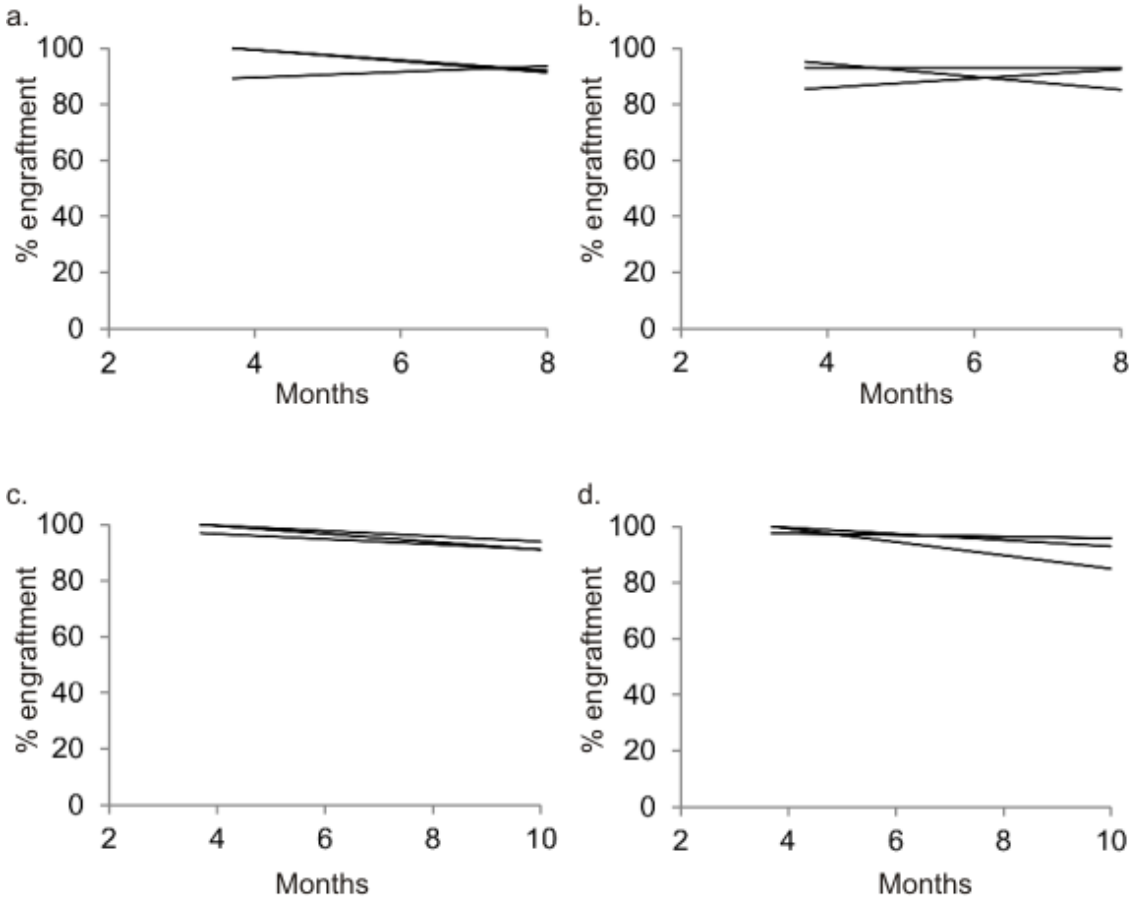

Figure 3.3 part 1 **Engraftment efficiency decreases over time in female ERα$^{+/+}$→ERα$^{-/-}$ chimeras**

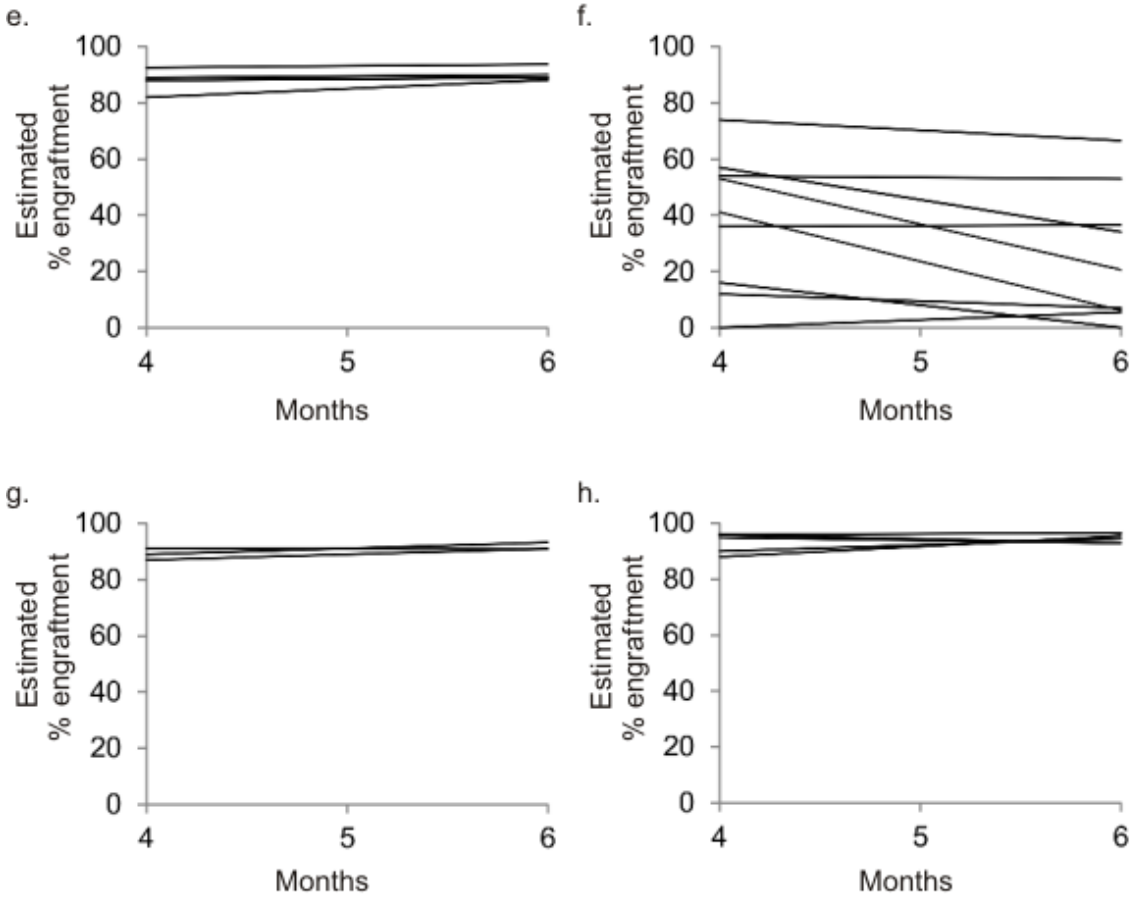

Figure 3.3 part 2 **Engraftment efficiency decreases over time in female ERα$^{+/+}$→ERα$^{-/-}$ chimeras**

Figure 3.3 **Engraftment efficiency decreases over time in female ERα$^{+/+}$→ERα$^{-/-}$ chimeras**

Engraftment efficiency was measured at later time points. The engraftment efficiency did not change significantly between 4 and 8 months in (a) female ERα$^{+/+}$→ERα$^{fl/+}$ chimeras, (b) female ERα$^{-/-}$→ERα$^{fl/+}$ chimeras, or between 4 and 10 months in (d) male ERα$^{+/+}$→ERα$^{fl/+}$ chimeras. In (c) ERα$^{-/-}$→ERα$^{fl/+}$ males engraftment decreased slightly but significantly between 4 and 10 months, at a rate of less than 2% per month (p=0.01). The engraftment of (f) female ERα$^{+/+}$→ERα$^{-/-}$chimeras decreased significantly between 4 and 6 months (p=0.03), but no decrease in engraftment occurred between 4 and 6 months in (e) female ERα$^{+/+}$→ERα$^{+/-}$ mice, (g) male ERα$^{+/+}$→ERα$^{+/-}$ mice, or (h) ERα$^{+/+}$→ERα$^{-/-}$male mice.

The engraftment efficiency of $ERα^{+/+}$→$ERα^{+/-}$ and $ERα^{+/+}$→$ERα^{-/-}$ chimeras was re-measured at 6 months of age. In female $ERα^{+/+}$→$ERα^{-/-}$ chimeras, there was a significant decrease in engraftment efficiency between 4 and 6 months of age (Figure 3.3f). On average, the amount of donor-derived DNA detected in these mice decreased by 14% over this period, although this amount varied by individual. Female $ERα^{+/+}$→$ERα^{+/-}$ and male $ERα^{+/+}$→$ERα^{-/-}$ and $ERα^{+/+}$→$ERα^{+/-}$ chimeras had stable engraftment, and there was no significant variation in engraftment between 4 month and 6 months (Figure 3.3e,g,h). Therefore, in female mice, loss of *ERα* in recipient mice transplanted with $ERα^{+/+}$ hematopoietic cells results in a progressive decrease in the proportion of $ERα^{+/+}$ hematopoietic cells. This effect is not observed in female $ERα^{-/-}$→$ERα^{fl/+}$ mice which also had lower engraftment efficiency 8 weeks after transplant compared to controls. In male mice, the *ERα* genotype of hematopoietic and non-hematopoietic cells does not cause a large progressive decrease in engraftment efficiency.

CXCR4, CXCR7, and CXCL12 Expression is Not Affected by ERα

The success of hematopoietic reconstitution was affected by the *ERα* genotype of hematopoietic cells and non-hematopoietic cells in female, but not male mice. The chemokine CXCL12 mediates localization of HSCs to the stem cell niche by interacting with the chemokine receptors CXCR4 and CXCR7 on hematopoietic cells. The expression of these is regulated by estrogen (Boudot, 2011; Li, 2013). A change in the expression of one or more of these molecules could be responsible for impaired hematopoietic reconstitution in female mice.

Therefore, we examined the expression of CXCL12, CXCR4, and CXCR7 in global $ERα^{+/+}$ and $ERα^{-/-}$ mice. RNA was isolated from the bone marrow of $ERα^{+/+}$ and $ERα^{-/-}$ mice, and analyzed by QRT-PCR for CXCR4, CXCR7, and CXCL12, and compared to

GAPDH. We found no significant differences in the expression of CXCR4, CXCR7, or CXCL12 in bone marrow cells from *ERα*$^{+/+}$ and *ERα*$^{-/-}$ mice (Figure 3.4).

ERα in Hematopoietic Cells Does Not Affect Albuminuria or Survival

Although they do not all develop disease at exactly the same time, (NZB x NZW)F1 mice develop lupus at a predictable rate. Females develop and succumb to disease several months before males. *ERα*$^{+/+}$ females develop albuminuria with a median latency of 201 days, while *ERα*$^{+/+}$ males develop albuminuria at a median latency of 335 days (Bynote, 2008). The median lifespan for *ERα*$^{+/+}$ females is 238 days and for *ERα*$^{+/+}$ males is 321 days (Bynote, 2008). *ERα*$^{-/-}$ (NZB x NZW)F1 mice have significantly delayed albuminuria and prolonged survival. For female (NZB x NZW)F1 mice, loss of *ERα* increases the median survival time by >308 days, and median survival is extended by 113 days in males (Bynote, 2008). Therefore, loss of *ERα* has a greater impact on lupus in female mice.

In this study, albumin in the urine, or albuminuria, was measured as a non-invasive way to assess renal function. Albumin is a protein which is excluded from the urine by healthy kidneys, but is present in the urine of individuals with nephritis, and an increased concentration of albumin correlates with increased kidney damage. These chimeric mice were also monitored for survival until they reached a maximum of one year of age.

In ERα$^{-/-}$→ERα$^{fl/+}$ and ERα$^{+/+}$→ERα$^{fl/+}$ female mice with successful engraftment, there was no difference in the incidence and latency to albuminuria (Figure 3.5a). 83% of ERα$^{-/-}$→ERα$^{fl/+}$ and 92% of ERα$^{+/+}$→ERα$^{fl/+}$ females developed detectable albuminuria before death, at a median age of 234 and 244 days, respectively. Similarly, there was no difference in the survival of ERα$^{-/-}$→ERα$^{fl/+}$ and ERα$^{+/+}$→ERα$^{fl/+}$ females (Figure 3.5b). The median survival of ERα$^{-/-}$→ERα$^{fl/+}$ females was 258 days, which is not different from

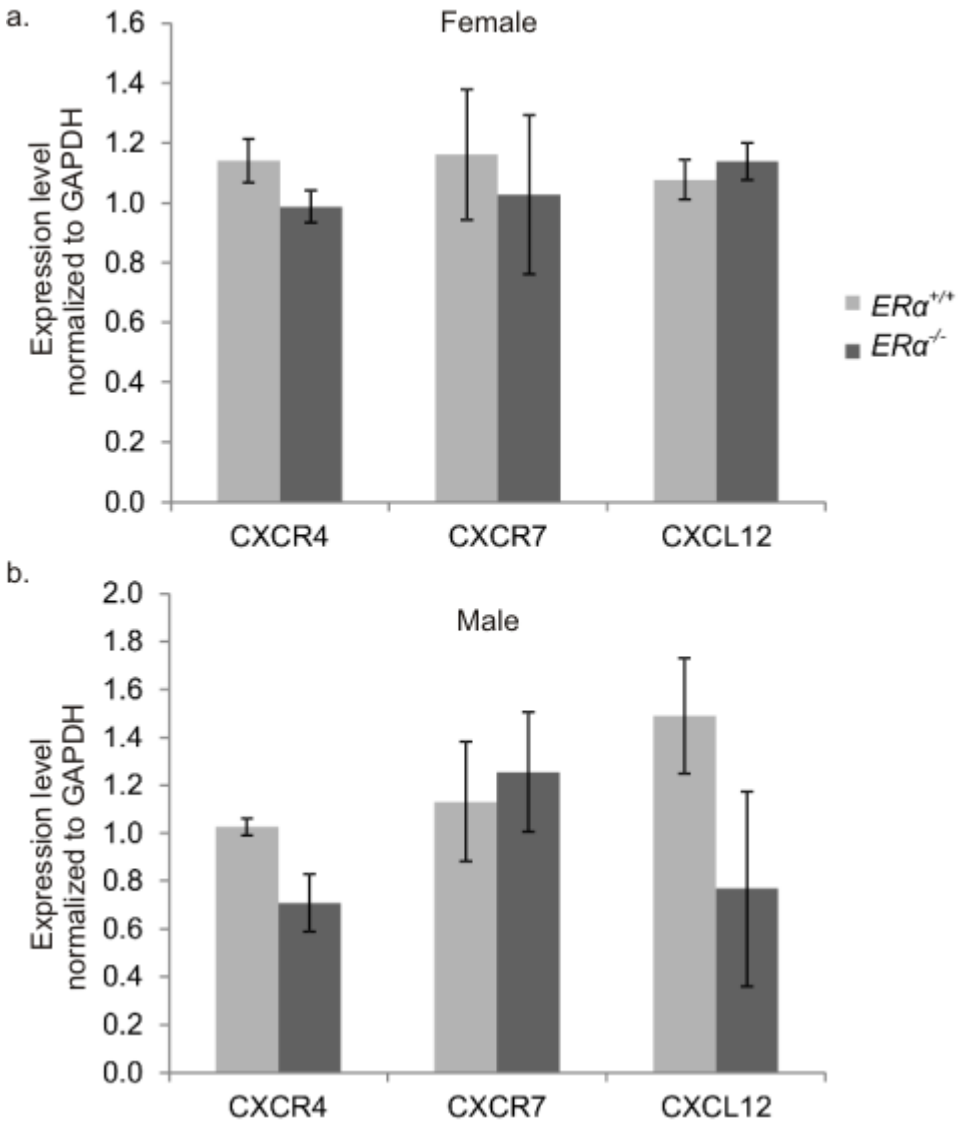

Figure 3.4 **Expression of chemokine receptors is not affected by *ERα* deficiency**

Figure 3.4 **Expression of chemokine receptors is not affected by *ERα* deficiency**

Levels of CXCR4, CXCR7, and CXCL12 mRNA were quantitatively measured in bone marrow cells from $ERα^{+/+}$ and $ERα^{-/-}$ mice. There were no significant differences in mRNA levels in (a) female or (b) male mice.

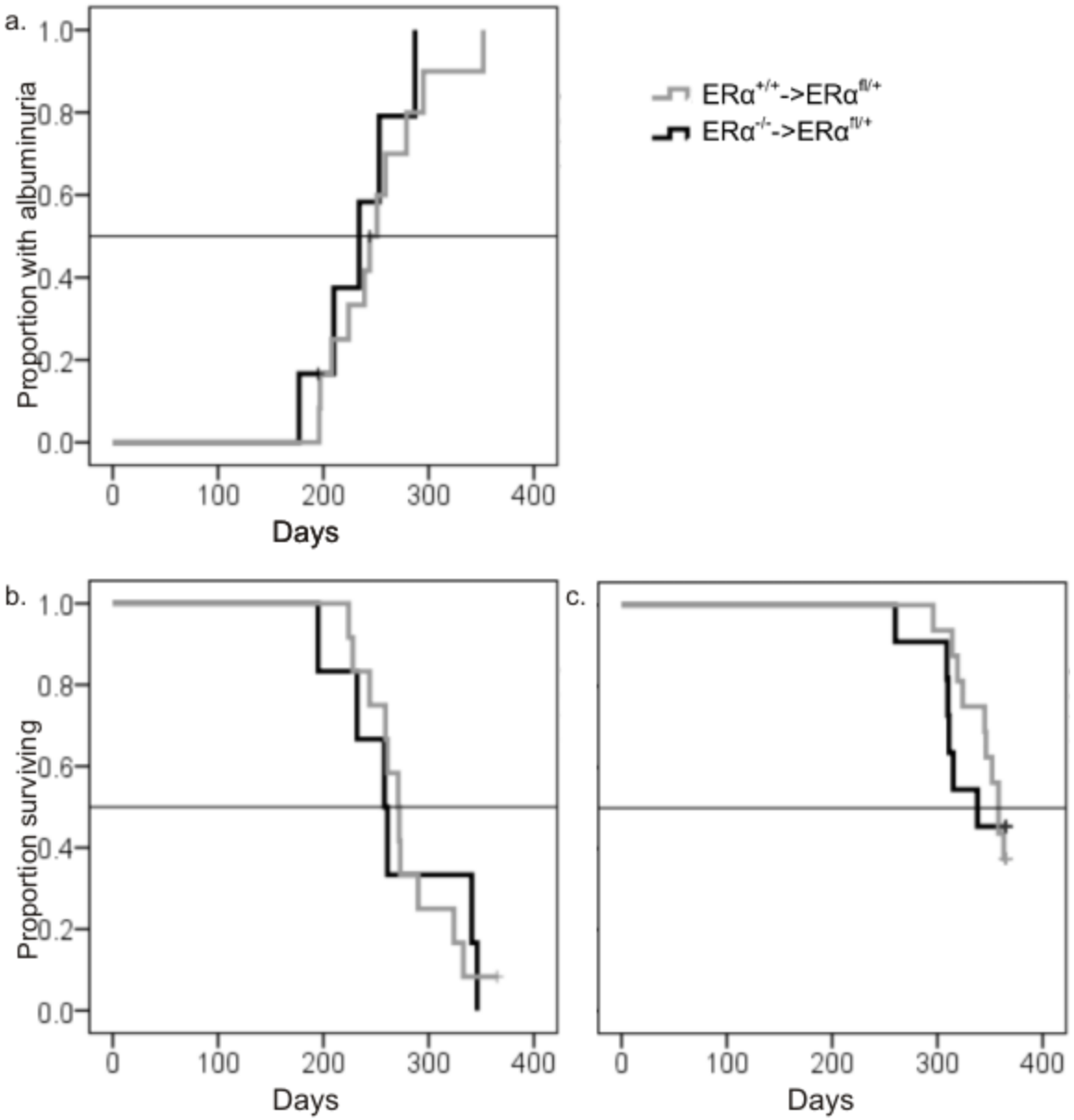

Figure 3.5 **The *ERα* genotype of hematopoietic cells does not impact the survival of (NZB x NZW)F1 chimeras**

Figure 3.5 **The *ERα* genotype of hematopoietic cells does not impact the survival of (NZB x NZW)F1 chimeras**

(a) Albuminuria was measured in female $ERα^{+/+}$→$ERα^{fl/+}$ and $ERα^{-/-}$→$ERα^{fl/+}$ mice, and no difference was found in the median latency to albuminuria. Survival was monitored for up to one year in (b) female and (c) male $ERα^{+/+}$→$ERα^{fl/+}$ and $ERα^{-/-}$→$ERα^{fl/+}$ mice, and there was no difference in the median survival in mice of either sex.

the 271 day median survival of $ERα^{+/+}→ERα^{fl/+}$ females. This suggests that, in female mice, the *ERα* genotype of hematopoietic cells does not affect the development of lupus.

Albuminuria and survival were initially monitored in $ERα^{+/+}→ERα^{+/-}$ and $ERα^{+/+}→ERα^{-/-}$ chimeric mice. However, after it became clear that the engraftment in $ERα^{+/+}→ERα^{-/-}$ females was uniformly unsuccessful, the albuminuria and survival studies were discontinued. All remaining mice in this arm of the study were sacrificed at six months of age.

No female mice from the $ERα^{+/+}→ERα^{-/-}$ group achieved successful engraftment, so these mice have a hematopoietic compartment which consists of a mixed population of $ERα^{+/+}$ and $ERα^{-/-}$ cells. When mice from this group were compared to $ERα^{+/+}→ERα^{+/-}$ mice, there was no difference in survival (Figure 3.6a). $ERα^{+/+}→ERα^{-/-}$ females had a median survival of 247 days, and $ERα^{+/+}→ERα^{+/-}$ females had a median survival of 278 days. Because the hematopoietic compartment of $ERα^{+/+}→ERα^{-/-}$ females is a mixed population, we cannot draw conclusions about the impact of *ERα* in non-hematopoietic cells on lupus with these mice.

Albuminuria was not accurately assessed in males, because I was not able to consistently express urine in male mice, so albuminuria data will not be presented for males, but the survival of males was monitored for up to one year. There was no difference in the survival of $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ male mice (Figure 3.5c). $ERα^{-/-}→ERα^{fl/+}$ males had a median survival time of 338 days, and $ERα^{+/+}→ERα^{fl/+}$ males had a median survival time of 358 days. This suggests that the *ERα* genotype of hematopoietic cells does not affect survival in male mice.

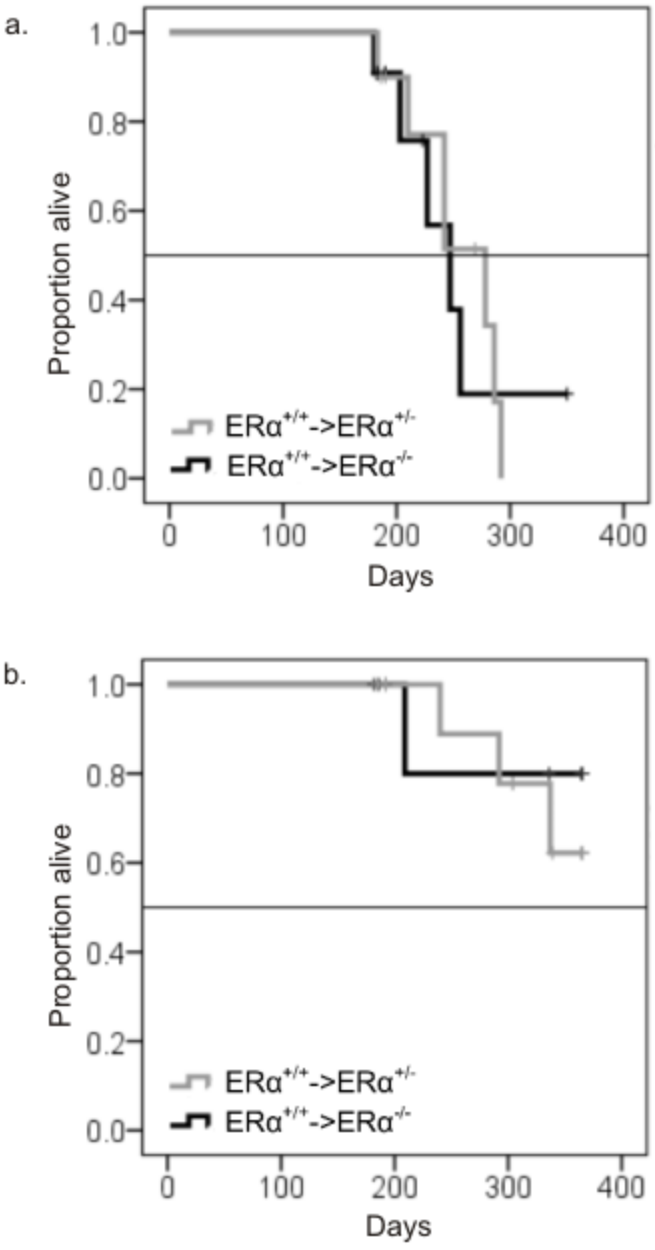

Figure 3.6 **The *ERα* genotype of non-hematopoietic cells does not impact the survival of chimeric (NZB x NZW)F1s**

Figure 3.6 **The *ERα* genotype of non-hematopoietic cells does not impact the survival of chimeric (NZB x NZW)F1s**

Survival was monitored for up to one year. In (a) female mice, including mice with inefficient engraftment, survival was not different in $ERα^{+/+}$→$ERα^{+/-}$ and $ERα^{+/+}$→$ERα^{-/-}$ mice. (b) There was no difference in the survival of $ERα^{+/+}$→$ERα^{+/-}$ and $ERα^{+/+}$→$ERα^{-/-}$ male mice.

There was no difference in the survival of $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ male mice (Figure 3.6b). Neither group of male mice reached 50% mortality over the course of this study, so the median survival could not be calculated for these groups.

Together, these survival studies suggest that $ERα^{-/-}$ hematopoietic cells are unable to attenuate lupus in female and male mice. Additionally, in male mice, no difference in survival was observed in mice with $ERα^{-/-}$ and $ERα^{+/+}$ non-hematopoietic cells, which suggests that *ERα* in non-hematopoietic cells does not affect the development of lupus.

Hematopoietic Estrogen Receptor Alpha Influences Autoantibody Production

(NZB x NZW)F1 lupus-prone mice produce antibodies against many nuclear antigens. To determine the effect of hematopoietic *ERα* expression on autoantibody production, $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ mice with successful engraftment were evaluated for autoantibody production using serum samples collected at monthly intervals. Non-pathogenic anti-chromatin IgG autoantibodies, which are among the first autoantibodies detected in lupus, were measured in female $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ mice at 5, 6, and 7 months of age. At 5 months of age, both groups produced very little anti-chromatin IgG and there was no difference in antibody production between these groups. However, at 6 months of age $ERα^{+/+}→ERα^{fl/+}$ females produced significantly more anti-chromatin IgG than $ERα^{-/-}→ERα^{fl/+}$ females (Figure 3.7a). At 7 months of age, there was no difference in the amount of anti-chromatin IgG produced by the two groups. In both groups of chimeric mice, there is a trend of increasing antibody production as mice age, which is consistent with previous observations in (NZB x NZW)F1 mice. These data suggest that female mice with $ERα^{-/-}$ hematopoietic cells have attenuated lupus at 6 months, but by 7 months this attenuation is overcome and immune activation is similar to females with $ERα^{+/+}$ hematopoietic cells.

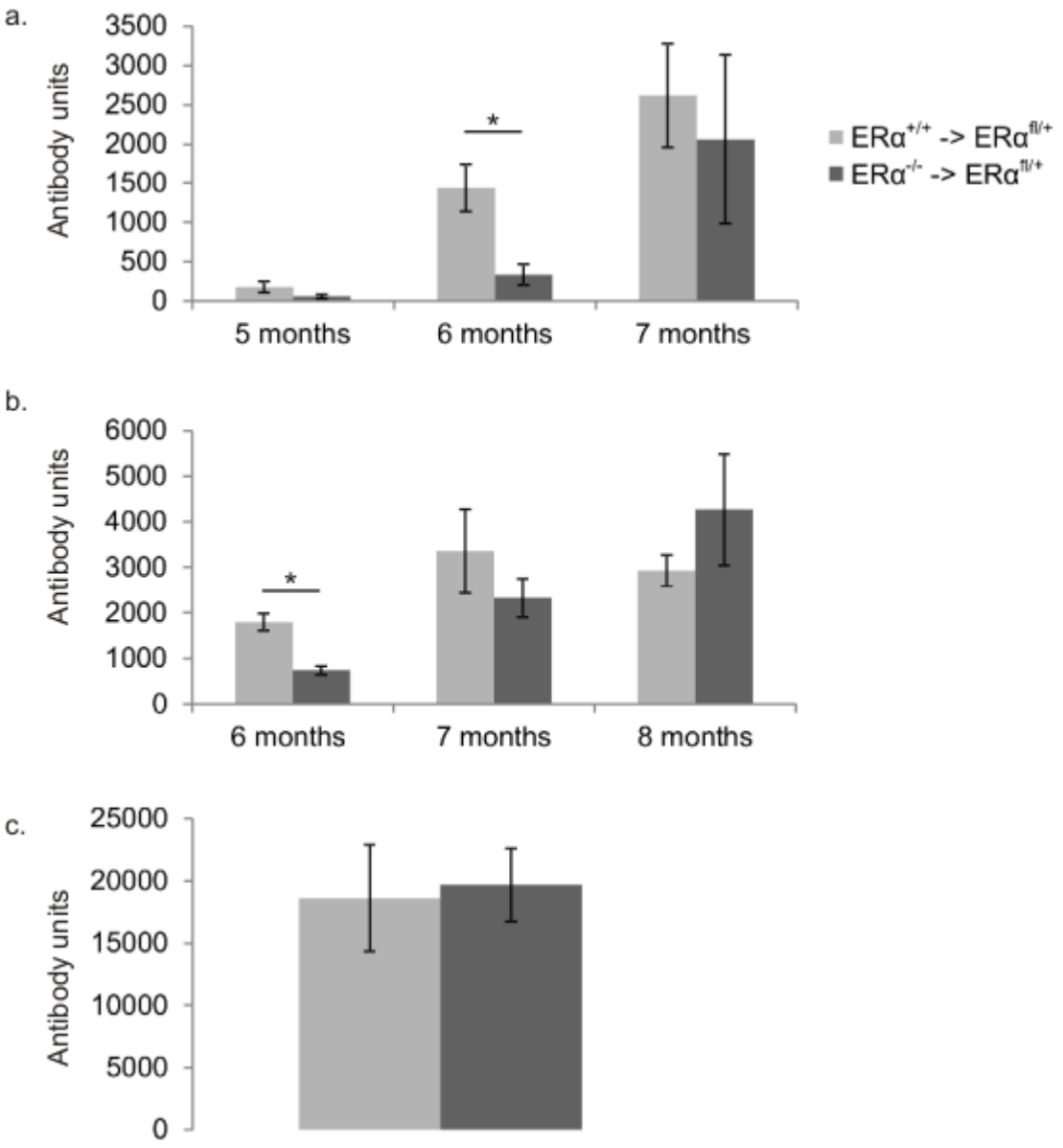

Figure 3.7 **Fewer autoantibodies are produced by female ERα$^{-/-}$ → ERα$^{fl/+}$ chimeras at 6 months of age**

Figure 3.7 **Fewer autoantibodies are produced by female ERα$^{-/-}$ → ERα$^{fl/+}$ chimeras at 6 months of age**

(a) Serum α-chromatin IgG was measured by ELISA in female ERα$^{+/+}$→ERα$^{fl/+}$ and ERα$^{-/-}$→ERα$^{fl/+}$ mice at 5, 6, and 7 months of age. ERα$^{-/-}$→ERα$^{fl/+}$ mice produced significantly less α-chromatin IgG at 6 months of age ($p=0.006$). (b) Anti-dsDNA IgG antibodies were measured in the same mice at 6, 7, and 8 months of age in the same mice. At 6 months ERα$^{-/-}$→ERα$^{fl/+}$ mice produced less anti-dsDNA IgG. (c) Anti-dsDNA IgG was measured in male mice at 8 months of age, and there was no difference in antibody production between ERα$^{+/+}$→ERα$^{fl/+}$ and ERα$^{-/-}$→ERα$^{fl/+}$ males.

Pathogenic anti-dsDNA IgG autoantibodies were measured at 6, 7, and 8 months of age in $ERα^{-/-}$→$ERα^{fl/+}$ and $ERα^{+/+}$→$ERα^{fl/+}$ females. Anti-dsDNA IgG production followed the same trend that was observed with anti-chromatin IgG production in these mice. At 6 months of age, $ERα^{+/+}$→$ERα^{fl/+}$ females produced significantly more anti-dsDNA IgG than $ERα^{-/-}$→$ERα^{fl/+}$ females (Figure 3.7b). However, by 7 and 8 months of age there was no difference in the anti-dsDNA IgG production between these groups. As with anti-chromatin IgG, anti-dsDNA IgG usually increases over time in (NZB x NZW)F1 mice. This was observed in these experimental mice until 8 months of age, by which time many of the sickest mice had died, and the average level of anti-dsDNA IgG plateaued. Therefore, female mice with $ERα^{-/-}$ hematopoietic cells produced less anti-dsDNA IgG at 6 months of age, but as these mice aged, autoimmunity was activated, and lupus was no longer attenuated.

The latency to death in chimeric mice is similar between females at 6 months of age, and males at 8 months of age; therefore, anti-dsDNA IgG production was measured in $ERα^{+/+}$→$ERα^{fl/+}$ and $ERα^{-/-}$→$ERα^{fl/+}$ males at 8 months of age. Male mice produce the same amount of this antibody at 8 months of age (Figure 3.7c).

B and T cell Populations Are Unaffected by Hematopoietic ERα

ERα affects the development of many types of immune cells. Plasma cells and activated B and T cells are cells associated with more aggressive lupus, while other immune cells, like Tregs, negatively regulate the immune response. We examined several lymphocyte populations in the bone marrow and spleens of chimeric mice to see if *ERα* expression in hematopoietic cells affected these populations. The cells for this experiment were collected when mice were removed from the survival study due to signs of illness. A large proportion of male mice survived until the end of the study without developing signs of illness. This, combined with the relatively small number (n = 15/ group) of mice

produced for these studies, resulted in a very small number of ill male mice. Therefore, we were not able to capture data from enough ill males to compare $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ mice. Almost all female mice showed signs of illness over the course of the study, so we were able to capture data from enough $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ female mice to compare these two groups.

No differences were found in the percentage of plasma cells, memory B cells, or Tregs in either the bone marrow or spleens of $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ female mice (Table 3.2). This indicates that at the end-stage of disease, the sizes of these B and T cell populations are not different in mice with and without *ERα* expression in hematopoietic cells.

One consequence of knockout of *ERα* in (NZB x NZW)F1 mice is a dramatic reduction in the activation of B and T cells (Gould lab, unpublished data). Activation of B and T cells was also examined in female $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ mice. There was no difference in the percentage of activated B cells or activated T cells in the spleen (Table 3.3). This indicates that, at least at the end stage of disease, $ERα^{-/-}$ and $ERα^{+/+}$ hematopoietic cells are similarly activated in (NZB x NZW)F1 chimeras.

ERα Does Not Impact the Development of Moderate to Severe Glomerulonephritis

In order to determine if the development of glomerulonephritis was affected by *ERα* in hematopoietic and non-hematopoietic cells, kidneys were collected at the end of the survival study. Kidneys sections were stained with Periodic acid-Schiff, and analyzed for signs of nephritis. All kidneys that were examined from $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ mice had moderate to severe glomerulonephritis (Figure 3.8a). There was no difference in percentage of affected glomeruli between $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ females; the mean percent of affected glomeruli was 78% and 80%,

Table 3.2 **Lymphocyte populations in chimeric mice**

	Bone marrow		**Spleen**	
	$ERα^{-/-}$→$ERα^{fl/+}$	**$ERα^{+/+}$→$ERα^{fl/+}$**	**$ERα^{-/-}$→$ERα^{fl/+}$**	**$ERα^{+/+}$→$ERα^{fl/+}$**
% Plasma cells	5.4 ± 1.3	8.0 ± 3.8	2.5 ± 0.3	2.4 ± 0.05
% Total memory B cells	3.2 ± 1.2	4.7 ± 1.3	5.4 ± 1.2	1.3 ± 0.3
% T regulatory cells	6.0 ± 2.0	8.2 ± 5.8	4.1 ± 2.0	6.4 ± 1.6

Plasma cells- % of live $CD138^+$ cells
Total memory B cells- % of $CD27^+$ of live $CD19^+$ cells
T regulatory cells- % of $CD25^+$ $CD62L^{lo}$ of live $CD4^+$ cells

Table 3.3 **B and T cell activation in the spleen**

	ERα$^{-/-}$→ERα$^{fl/+}$	**ERα$^{+/+}$→ERα$^{fl/+}$**
% Activated B cells	12.4 ± 2.1	13.0 ± 3.3
% Activated T cells	17.5 ± 4.8	18.9 ± 3.9

Activated B cells- % of CD80/86^{+} of live CD19^{+} cells
Activated T cells- % of CD69^{+} of live CD4^{+} B cells

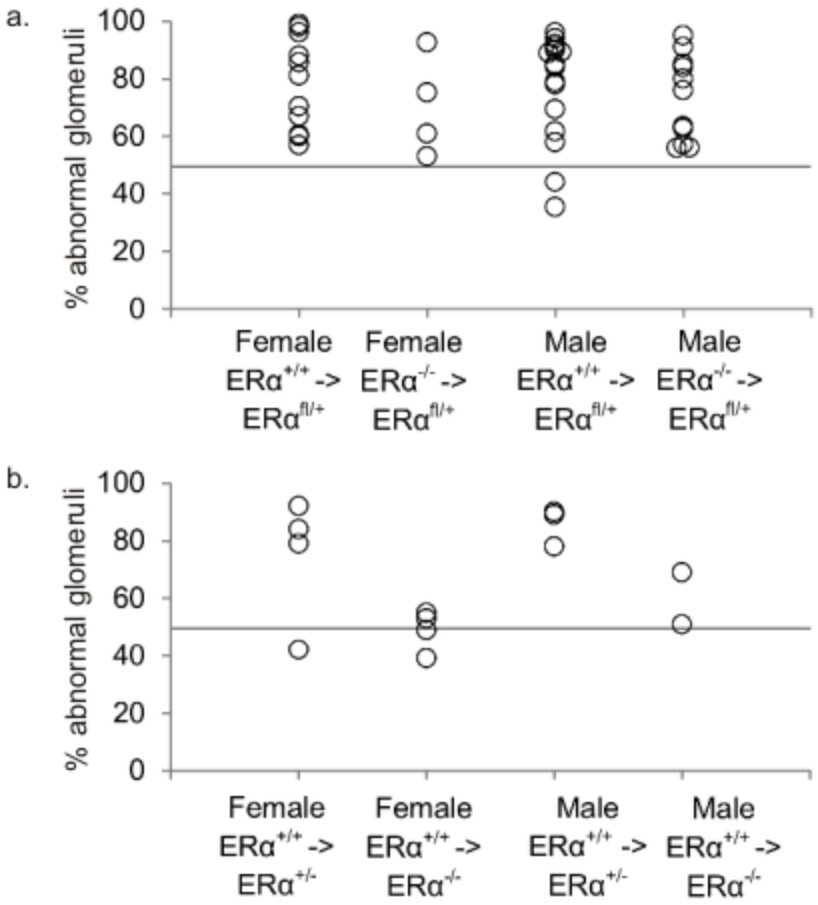

Figure 3.8 part 1 **Chimeric (NZB x NZW)F1 mice develop severe glomerulonephritis at the same frequency**

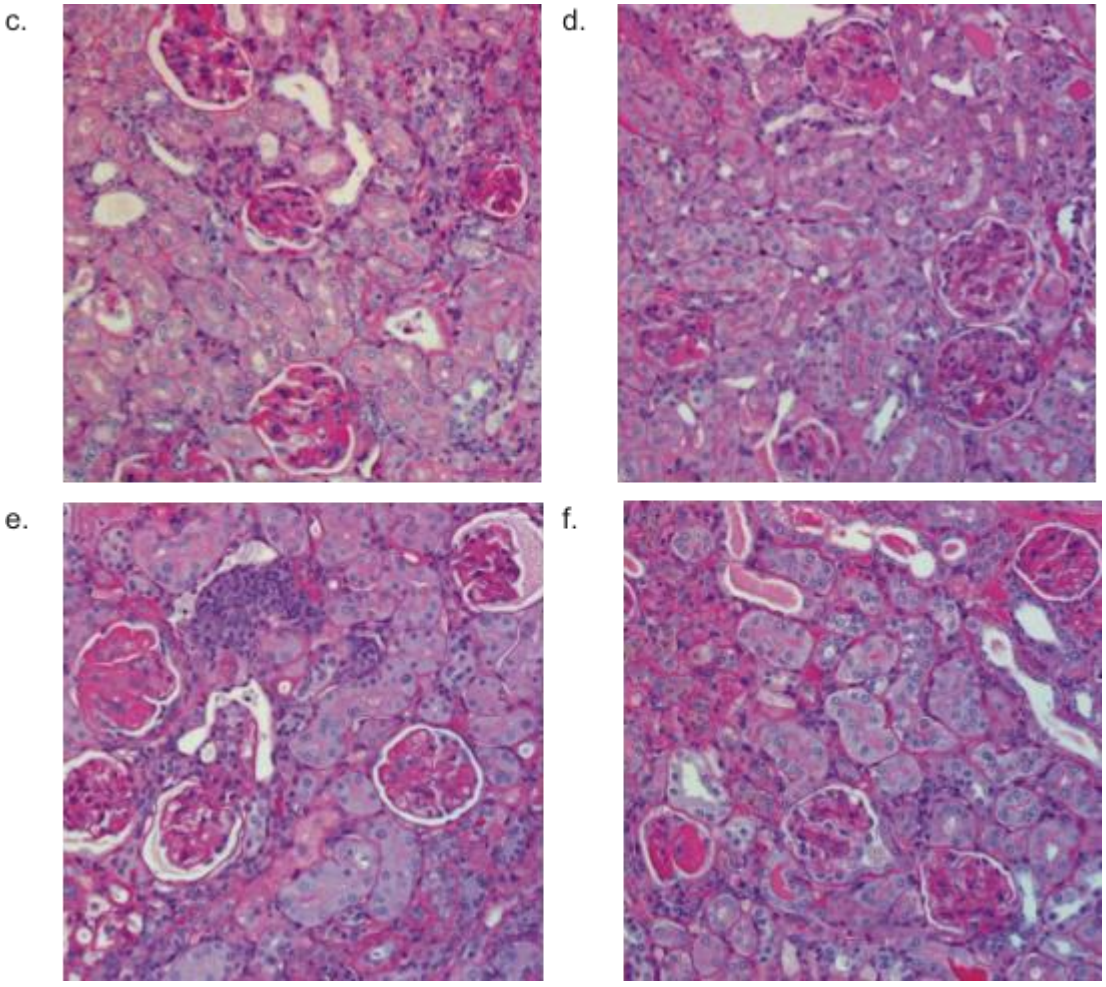

Figure 3.8 part 2 **Chimeric (NZB x NZW)F1 mice develop severe glomerulonephritis at the same frequency**

Figure 3.8 **Chimeric (NZB x NZW)F1 mice develop severe glomerulonephritis at the same frequency**

Kidneys collected at the end of the survival study were stained with PAS and evaluated for glomerulonephritis. (a) Almost all kidneys from the $ERα^{+/+}→ERα^{fl/+}$ and $ERα^{-/-}→ERα^{fl/+}$ chimeras had severe glomerulonephritis and those that did not had moderate glomerulonephritis. (b) Although few mice in the $ERα^{+/+}→ERα^{+/-}$ and $ERα^{+/+}→ERα^{-/-}$ groups were kept until the end of the survival study, the kidneys from these mice all had moderate or severe glomerulonephritis. Representative sections from (c) female $ERα^{+/+}→ERα^{fl/+}$, (d) female $ERα^{-/-}→ERα^{fl/+}$, (e) male $ERα^{+/+}→ERα^{fl/+}$, and (f) male $ERα^{-/-}→ERα^{fl/+}$ chimeras are shown.

respectively. Similarly, there was no difference in the percentage of affected glomeruli in males. Male ERα$^{+/+}$→ERα$^{fl/+}$ mice had an average of 77% affected glomeruli, while ERα$^{-/-}$→ERα$^{fl/+}$ males had an average of 74% affected glomeruli. Representative images of PAS-stained kidneys from each of these groups are shown (Figure 3.8c-f).

Because the survival studies for ERα$^{+/+}$→ERα$^{+/-}$ and ERα$^{+/+}$→ERα$^{-/-}$ mice were terminated prematurely, there were few samples available from mice that had completed the survival study in which to analyze glomerulonephritis. However, 2-4 samples per group were analyzed, and all showed signs of moderate to severe glomerulonephritis (Figure 3.8b). All types of chimeric mice from these studies developed glomerulonephritis characteristic of lupus in (NZB x NZW)F1 mice. Therefore, these mice are dying from lupus-related causes, not as a side effect of the irradiation and transplantation procedure.

Autoreactive Plasma Cells Survive Irradiation

ELISA data indicated that the development of autoimmunity is delayed in ERα$^{-/-}$→ERα$^{fl/+}$ females compared to ERα$^{+/+}$→ERα$^{fl/+}$ females, which supported our hypothesis that *ERα* in immune cells promotes lupus. However, the subsequent increase in autoantibody production suggested that somehow *ERα*$^{-/-}$ immune cells became activated and were driving the progression of lupus. If *ER*$^{fl/+}$ host-derived plasma cells were driving autoantibody production in 7 and 8 month old ERα$^{-/-}$→ERα$^{fl/+}$ female mice, this could also be the reason that we did not observe a difference in survival between mice with *ERα*$^{-/-}$ and *ERα*$^{+/+}$ hematopoietic cells.

In an effort to address the hypothesis that the failure to see attenuation of lupus in ERα$^{-/-}$→ERα$^{fl/+}$ mice was due to antibody production by residual, host-derived *ERα*$^{fl/+}$ plasma cells, we looked for the presence of *ERα*$^{fl/+}$ plasma cells in mice at the end of the

survival study. $CD138^+$ plasma cells were isolated with a magnetic column, DNA was isolated, and QPCR used to detect the amount of DNA derived from host plasma cells. DNA from the $ER\alpha^{fl/+}$ host could be detected in 60% of plasma cell samples from bone marrow and 62.5% from spleen (Figure 3.9a). Overall, $ER\alpha^{fl/+}$ DNA was detected in 6 of 9 mice used for this experiment from both $ER\alpha^{+/+} \rightarrow ER\alpha^{fl/+}$ and $ER\alpha^{-/-} \rightarrow ER\alpha^{fl/+}$ mice. All of the mice in which $ER\alpha^{fl/+}$ plasma cell DNA was not detected were one year old, so it is possible that $ER\alpha^{fl/+}$ plasma cells could have been detected in these mice at a younger age. This indicates that long-lived plasma cells may survive irradiation, and persist for 7-10 months after irradiation.

Although detection of $ER\alpha^{fl/+}$ plasma cells in chimeric mice indicated that some host plasma cells either survived irradiation or arose from cells that did, this experiment did not address the specificity of these cells. To determine if host dsDNA-reactive plasma cells survived irradiation, young (NZB x NZW)F1 mice were irradiated and not injected with replacement bone marrow. After one week, plasma cells were isolated and anti-dsDNA IgG ELISpot was performed. Figure 3.9b shows an example of ELISpot wells containing blue-green spots which represent dsDNA-reactive plasma cells.

Irradiation of young mice caused a greater than ten-fold decrease in the number of live cells recovered from the bone marrow and spleen. Accordingly, after one week the spleen weight was reduced by 65-75% in irradiated mice compared to unirradiated littermate controls. Live long-lived plasma cells (PI^- $CD138^+$ $MHCII^{lo}$) and short-lived plasma cells (PI^- $CD138^+$ $MHCII^{hi}$) were isolated from the bone marrow and spleen and applied to a plate coated with dsDNA.

dsDNA-reactive long-lived and short-lived plasma cells were detected in the spleen and bone marrow of young, irradiated mice. The percent of dsDNA-reactive cells ranged

from 0.27-0.41% (Table 3.4). This is a relatively high percentage of plasma cells to be reactive to a single antigen. These results show that pathogenic dsDNA-reactive plasma cells can survive irradiation in 10 week old (NZB x NZW)F1 mice. These residual pathogenic cells may be sufficient to cause lupus in these mice.

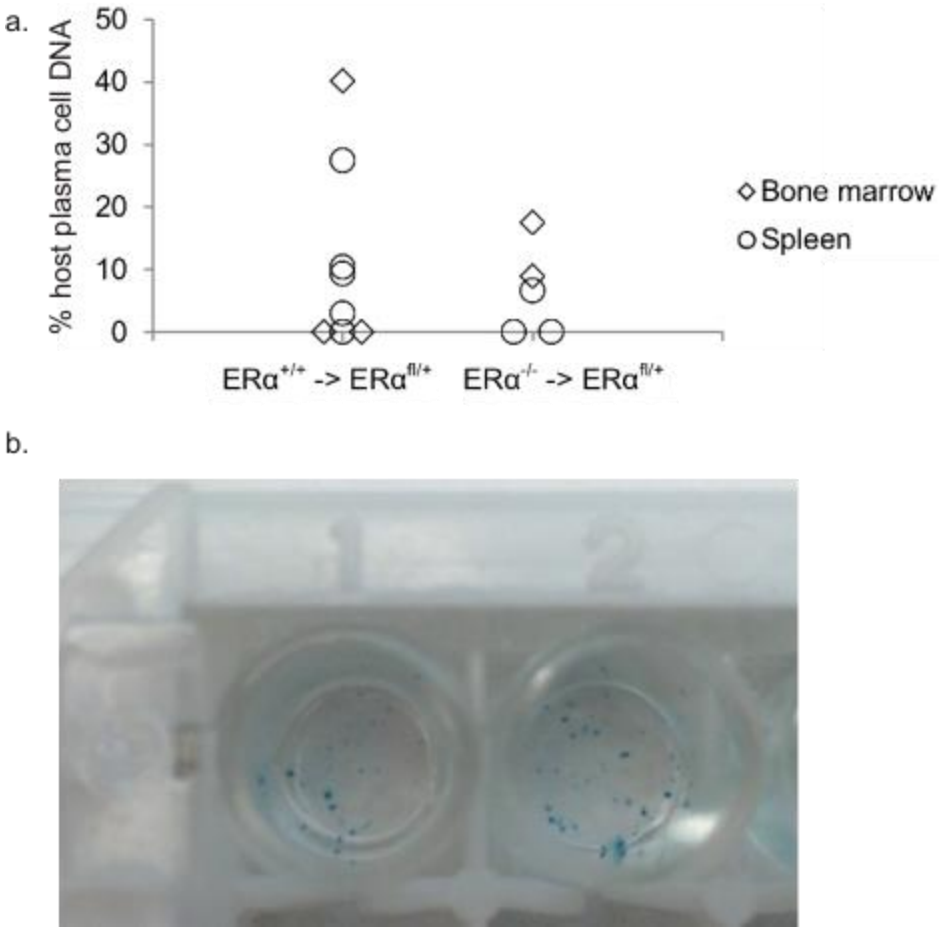

Figure 3.9 **Autoreactive plasma cells from the host survive irradiation**

Figure 3.9 **Autoreactive plasma cells from the host survive irradiation**

Plasma cells were isolated from ERα$^{+/+}$→ERα$^{fl/+}$ and ERα$^{-/-}$→ERα$^{fl/+}$ mice at the end of the survival study, and the origin of the cells was evaluated by QPCR. (a) Plasma cells from host mice were detected in the bone marrow and spleen of mice which had received both *ERα*$^{+/+}$ and *ER*$^{-/-}$ transplants. (b) An example of an ELISPOT plate showing dsDNA-reactive plasma cells.

Table 3.4 **dsDNA-reactive plasma cells survive irradiation in young (NZB x NZW)F1 mice**

	Long-lived plasma cells	Short-lived plasma cells
Spleen	0.27 ± 0.07%	0.41 ± 0.29%
Bone marrow	0.37 ± 0.09%	0.41 ± 0.07%

Long-lived plasma cells (PI^- $CD138^+$ $MHCII^{lo}$)
Short-lived plasma cells (PI^- $CD138^+$ $MHCII^{hi}$)

Discussion

ERα signaling promotes the pathogenesis of lupus in (NZB x NZW)F1 mice (Bynote, 2008; Li, 2007). To investigate the cell type/types that mediate this effect, we created chimeric (NZB x NZW)F1 mice with *ERα*$^{-/-}$ hematopoietic cells or *ERα*$^{-/-}$ non-hematopoietic cells. Although our studies were not able to delineate the impact of *ERα*$^{-/-}$ hematopoietic and non-hematopoietic cells on lupus, our studies demonstrated the importance of ERα signaling on hematopoietic reconstitution.

Age- and sex-matched (NZB x NZW)F1 *ERα*$^{+/+}$ and *ERα*$^{-/-}$ bone marrow was transplanted into (NZB x NZW)F1 *ERα*$^{fl/+}$ mice to examine the impact of the *ERα* genotype of hematopoietic cells on lupus. Among female mice, ERα$^{-/-}$→ERα$^{fl/+}$ chimeras have significantly less successful grafts than ERα$^{+/+}$→ERα$^{fl/+}$ chimeras, but grafts from both these sets of mice were stable over time. There was no difference in the engraftment efficiency between ERα$^{+/+}$→ERα$^{fl/+}$ and ERα$^{-/-}$→ERα$^{fl/+}$ male mice. Therefore, *ERα*$^{-/-}$ hematopoietic cells from female and male mice have different potential for engraftment. This difference is caused by intrinsic differences between cells from females and males. HSCs from females require *ERα* to maintain the normal rate of cell division (Nakada, 2014). *ERα*$^{-/-}$ cells from females could also have an impaired ability to hone to the stem cell niche.

In a set of complementary experiments, *ERα*$^{+/+}$ bone marrow was transplanted to *ERα*$^{+/-}$ or *ERα*$^{-/-}$ mice in order to determine the impact of *ERα* in non-hematopoietic cells on lupus in (NZB x NZW)F1 mice. At 8 weeks after transplant, ERα$^{+/+}$→ERα$^{-/-}$ female mice have dramatically lower engraftment efficiency compared to ERα$^{+/+}$→ERα$^{+/-}$ females, and the efficiency of engraftment in ERα$^{+/+}$→ERα$^{-/-}$ females decreases over time. We believe that this is caused by an exhaustion of *ERα*$^{+/+}$ hematopoietic cells in *ERα*$^{-/-}$ female mice where serum E2 levels are 10x higher than in *ERα*$^{+/+}$ females. Treatment with estrogen

causes an increased rate of HSC and MPP division in a cell intrinsic manner (Nakada, 2010; Illing, 2012). This estrogen-induced increase in division is mediated by *ERα* (Nakada, 2010). Additionally, extended treatment with high dose E2 causes exhaustion of $ERα^{+/+}$ hematopoietic cells, while $ERα^{-/-}$ cells are resistant to this effect (Thurmond, 2000). Together, this supports our theory that the progressive decrease in engraftment efficiency in $ERα^{+/+} \rightarrow ERα^{-/-}$ female chimeric mice is due to exhaustion of $ERα^{+/+}$ hematopoietic cells caused by the high level of serum E2 in female $ERα^{-/-}$ mice. Exhaustion of $ERα^{+/+}$ hematopoietic cells causes $ERα^{-/-}$ cells that survived irradiation to comprise an increasing proportion of the hematopoietic population in these mice.

We hypothesized that another factor that may contribute to engraftment failure in $ERα^{+/+} \rightarrow ERα^{-/-}$ female mice may be a difference in the bone marrow microenvironment. A difference in the bone marrow microenvironment between female $ERα^{+/+}$ and $ERα^{-/-}$ mice could prevent HSCs from locating and engrafting in the stem cell niche. Our analysis of chemokine receptors CXCR4 and CXCR7, and the chemokine ligand CXCL12, which are involved in HSC homing to the stem cell niche, did not reveal a difference in the expression of these molecules between $ERα^{-/-}$ and $ERα^{+/+}$ female mice. Estrogen also has bone marrow stromal cell intrinsic effects, which affect the development of hematopoietic cells (Smithson, 1995). Therefore, $ERα^{-/-}$ bone marrow stromal cells could negatively impact the survival of $ERα^{+/+}$ hematopoietic cells.

No difference in engraftment success was observed in (NZB x NZW)F1 male mice from either set of transplants. This result was not surprising, because ERα does not have the same effects on hematopoietic cells in males that it has in females. HSCs divide at the same rate in $ERα^{+/+}$ and $ERα^{-/-}$ male mice (Nakada, 2014). Additionally, male $ERα^{-/-}$ mice have the same level of serum E2 as $ERα^{+/+}$ males, so $ERα^{+/+}$ hematopoietic cells would not undergo rapid E2-induced division, as in females.

In female and male mice that were considered successful transplants, we found that the *ERα* genotype of hematopoietic cells does not impact median survival. The median survival times of $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ females are not different from each other, but are 20-33 days longer than global $ERα^{+/+}$ females (Bynote, 2008). Similarly, the median survival of male $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ chimeras are not different, but are 17-37 days longer than global $ERα^{+/+}$ males. The delay in lupus-related death observed in chimeras compared to non-transplanted (NZB x NZW)F1s likely occurs because, although the donor bone marrow cells are age-matched with the recipients, reconstitution of the hematopoietic compartment takes several weeks, and the pathogenic processes that promote lupus are delayed while reconstitution takes place.

Female $ERα^{-/-}→ERα^{fl/+}$ chimeras had lower levels of anti-chromatin and anti-dsDNA antibodies at 6 months of age (2.5-3 months before death) compared to $ERα^{+/+}→ERα^{fl/+}$ females. However, the amount of anti-chromatin and anti-dsDNA antibodies was not significantly different between these groups at 7 months of age. This suggests that although $ERα^{-/-}$ hematopoietic cells attenuate lupus at a young age, this effect is overcome in older mice, leading to increased autoantibody production.

The unsuccessful engraftment of $ERα^{+/+}→ERα^{-/-}$ female mice prevented us from assessing the impact of *ERα* in non-hematopoietic cells on lupus in female mice. These mice had hematopoietic compartments composed of a mix of $ERα^{+/+}$ and $ERα^{-/-}$ cells. There was no difference in the survival of male $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ mice, which suggested that the *ERα* genotype of non-hematopoietic cells does not impact the development of lupus.

A confounding factor that should be considered when interpreting these results is the incomplete ablation of the host immune system. Although irradiation effectively kills rapidly dividing cells, long-lived plasma cells are resistant to irradiation-induced cell death and can survive for more than 6 months after irradiation (Miller, 1967). A recent study has shown that many long-lived plasma cells are produced in young (NZB x NZW)F1 mice (> 10^5 by 6 weeks of age in the spleen) many hundreds of which are dsDNA-reactive (Taddeo, 2015).

For $ER\alpha^{-/-} \rightarrow ER\alpha^{fl/+}$ and $ER\alpha^{+/+} \rightarrow ER\alpha^{fl/+}$ chimeras, mice with up to 5% of host DNA in the peripheral blood were considered to have successful engraftment. Our ELISpot analysis showed that 0.27-0.41% of plasma cells were dsDNA reactive in 10 week old (NZB x NZW)F1 mice that express *ERα*. This seems to be quite a large percentage of plasma cells to be reactive against a single antigen. There have been few reports of the percentage of plasma cells reactive to a single antigen, likely because the frequency of these cells is very low. A study of 5-7 month old (NZB x NZW)F1s found that ~0.03% of bone marrow plasma cells were dsDNA reactive, while ~0.07% of spleen plasma cells were dsDNA reactive (Winter, 2015). Therefore, our analysis found that young, irradiated (NZB x NZW)F1 mice had a larger proportion of dsDNA-reactive plasma cells compared to older, unirradiated (NZB x NZW)F1 mice. Thus, we propose that the host-derived *ERα*-competent dsDNA-reactive cells significantly accelerated the development of lupus in (NZB x NZW)F1 chimeric mice.

Chapter 4: Deletion of ERα in B cells attenuates lupus in (NZB x NZW)F1 mice

Abstract

Lupus is a systemic autoimmune disease that disproportionally affects women and is characterized by the production of autoreactive antibodies against nuclear antigens. Estrogen has long been believed to play an important role in the pathogenesis of lupus. Knockout of *ERα* considerably ameliorates lupus in lupus-prone mice. Because of the significant pathogenic role of B cells in lupus, we hypothesized that *ERα* expression in B cells promotes lupus. To test this hypothesis, we generated a murine lupus model where *ERα* is deleted from B cells using a cre-loxP system on the (NZB x NZW)F1 genetic background. On this genetic background, the B cell specific CD19-Cre driver induced only moderately efficient deletion of *ERα*. Despite this, mice with B cell specific *ERα* deletion, albeit incomplete, survived significantly longer than controls, and produced significantly lower levels of pathogenic anti-dsDNA autoantibodies. B cell specific deletion of *ERα* did not disturb B cell populations in the bone marrow or spleen, but it caused a significant reduction in the proportion of B cells that were activated. Additionally, we found that the *CD19-Cre* knockin allele had a significant impact on lupus and B cell populations. This emphasizes the need for cre-only controls in experiments using cre-loxP systems.

Background

Lupus is a disease in which multiple types of hematopoietic cells function abnormally in ways that lead to the development of systemic autoimmunity. Although multiple types of hematopoietic cells contribute to lupus, B cells play a central role. Lupus patients, as well as lupus-prone mice, have autoreactive B cells that produce autoantibodies. Antibodies against dsDNA are pathogenic in lupus. In a lupus-prone mouse strain that lacks mature B cells, the lupus phenotype is completely ameliorated (Shlomchik, 1994). A mild form of lupus occurs when the same strain of lupus-prone mice produce B cells that do not secrete antibody (Chan, 1999).

Depletion of B cells is an attractive therapeutic target for the treatment of lupus, but disappointingly, B cell depletion with the CD20 antibody rituximab did not significantly improve symptoms in lupus patients. In 2011, belimumab became the first new FDA approved lupus treatment in 56 years. Belimumab is an antibody that restricts the number of B cells produced by depleting the B cell growth factor BAFF, which is required for the survival of developing B cells. Despite the low proportion of patients who showed significant symptom improvement from belimumab treatment, its approval by the FDA demonstrates both the great need for new lupus therapies and the potential for B cell targeting by future lupus therapies.

Lupus is a disease that predominately affects young women, and it has long been thought that estrogens play a key role in the development of lupus. Exposure to both endogenous and exogenous estrogens is a risk factor for developing lupus (Costenbader, 2007). In lupus-prone mice, estrogen signaling through ERα significantly promotes autoantibody production and mortality (Li, 2007; Bynote, 2008). In these studies, the cell type or types responsible for these effects could not be identified.

Estrogens have many effects on the immune system, and particularly impact B cells in multiple ways which contribute to the development of autoimmunity.

Immature B cells in the bone marrow which have strongly autoreactive BCRs normally undergo clonal deletion. In the periphery, autoreactive mature B cells are rendered anergic at the transitional stage in secondary lymphoid organs. High levels of E2 allow high-affinity autoreactive B cells to escape negative selection at the immature and T2 stages (Grimaldi, 2006). Additionally, in a high-estrogen environment, estrogen signaling in B cells, predominately through ERα, causes upregulation of *CD22* and *SHP-1* which leads to decreased BCR signaling, thereby increasing the concentration of antigen required for tolerization of autoreactive B cells, and protecting autoreactive B cells from receptor-mediated apoptosis (Grimald, 2002; Hill, 2011). High levels of E2 also cause increased expression of anti-apoptotic *Bcl-2* and the B cell survival factor *BAFF* (Grimaldi, 2002; Hill, 2011). By decreasing the strength of BCR signaling and increasing the expression of pro-survival molecules, estrogens enhance the survival of high-affinity autoreactive B cells.

In addition to autoreactive B cells that arise as the result of VDJ recombination, autoreactive B cells can be generated by somatic hypermutation in the periphery. Estrogen promotes somatic hypermutation by stimulating the expression of activation-induced deaminase (Mai, 2010; Pauklin, 2009). Therefore, estrogen can potentially promote the development of autoreactive B cells via multiple pathways.

Enhanced survival of autoreactive B cells in mice treated with high levels of E2 causes an increase in the marginal zone B cell population in the spleen (Grimaldi, 2001; Grimaldi, 2006). In lupus-prone mice, the spleen marginal zone B cell population is enriched for dsDNA-reactive B cells compared to follicular B cells (Enghard, 2011).

Additionally, *ERα* promotes the activation of splenic B cells in female mice that carry a (NZB x NZW)F1-derived lupus susceptibility loci (Yoachim, 2015).

Together, these data led us to hypothesize that ERα signaling in B cells promotes lupus. To test this hypothesis, we have generated lupus-prone (NZB x NZW)F1 mice with *ERα* deletion in the B cell compartment. (NZB x NZW)F1 lupus-prone mice have several important similarities to lupus patients. (NZB x NZW)F1 mice produce pathogenic anti-dsDNA antibodies and develop fatal glomerulonephritis. They also have a gender bias, with female mice developing and succumbing to disease several months before males. To develop (NZB x NZW)F1 mice with B cell specific *ERα* deletion, we created NZB females that carry the *CD19-Cre* knockin allele and are globally heterozygous for *ERα*. These females were bred to NZW males homozygous for the floxed allele of *ERα*. B cells begin to express *CD19* at the pro-B cell stage in the bone marrow. *CD19* expression causes deletion of the floxed *ERα* allele in B cells, while *ERα* is left intact in non-B cells. Global $ERα^{+/-}$ does not impact the survival or development of glomerulonephritis in (NZB x NZW)F1 mice (Bynote, 2008).

Previously, it has been shown that the deletion efficiency of the *CD19-Cre* knockin allele is 75-80% in bone marrow B cells and 90-95% in spleen B cells on the 129 genetic background (Rickert, 1997). Although the *CD19-Cre* knockin allele has been extensively used in other mouse strains, to our knowledge, this is the first report of its use in a lupus-prone mouse model. Our studies show that deletion of *ERα* in a moderate proportion of B cells caused significant amelioration of lupus in (NZB x NZW)F1 mice.

Methods

Production of experimental animals

To produce NZB mice carrying the CD19-Cre knockin allele, B6.129P2(C)-*Cd19*$^{tm1(cre)Cgn/}$J mice were purchased (The Jackson Laboratory, Bar Harbor, ME, USA) and crossed with NZB mice. Genotyping for the *CD19-Cre* knockin allele was done with (IMR1084Tg F:5'-GCGGTCTGGCAGTAAAAACTATC-3' and IMR1085Tg R: 5'-GTGAAACAGCATTGCTGTCACTT-3') primers, with (COO3IC F: 5'-CTAGGCCACAGAATTGAAAGATCT-3' and COO4IC R 5'-GTAGGTGGAAATTCTAGCATCATCC-3') primers as an internal positive control. Offspring carrying the CD19-Cre knockin allele (*CD19-Cre*) were backcrossed to NZB mice for 4-5 more generations using simple sequence length polymorphism (SSLP) marker assisted selection. NZB.CD19$^{Cre/+}$ mice were then crossed to NZB.ERα$^{+/-}$ mice previously generated in our lab, producing NZB mice heterozygous for both *CD19-Cre* and *ERα* (Bynote, 2008). Genotyping for the exon 2 deletion of *ERα* was performed as described (www.jax.org/protocols). The ERaEx2 F primer: 5'-TACGGCCAGTCGGGCATC-3' (0.5 uM/rxn) and either the ERaEx2wtR 5'-GTAGAAGGCGGGAGGGCCGGTGTC-3' (0.06 uM/rxn) or ERaEx2null R 5'-GCTACTTCCATTTGTCACGTCC-3' (2 uM/rxn) primers were used to produce 234 bp and ~300 bp products, respectively. NZW.ERα$^{fl/fl}$ mice homozygous for an *ERα* allele in which exon 3 is flanked by loxP sites, were previously produced by backcrossing the floxed *ERα* allele from B6.*ERα*$^{f/fl}$ mice to the NZW background using marker assisted selection, and intercrossing NZW.*ERα*$^{fl/+}$ mice as previously described (Nelson, 2016). Genotyping for the *ERα*^{fl} allele was done with the primers N6delcKF: 5'-GACTCGCTACTGTGCCGTGTGC-3' and N6del3R 5'-CTTCCCTGGCATTACCACTTCTCCT-3'. The *ERα*$^{+}$ allele produces a 275 bp product,

while the *ERα*^{fl} allele produces a 475 bp product. To produce experimental mice, NZB.CD19$^{Cre/+}$; ERα$^{+/-}$ female mice were crossed with NZW.ERα$^{fl/fl}$ males.

In addition to marker assisted selection performed in our lab, the genetic backgrounds of NZB.CD19$^{Cre/+}$ and NZW.ERα$^{fl/fl}$ mice were analyzed at the DartMouse Mouse Speed Congenic Core Facility at Dartmouth Medical School. Using a 1449 SNP Illumina beadchip, the genetic origins of 733 SNPs spread throughout the genome were determined.

All animals were housed under conditions of controlled humidity, temperature, and lighting in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the *Guide for the Care and Use of Laboratory Animals*. Mice had *ad libitum* access to 7904 Teklad Irradiated S-2335 Mouse Breeder Diet (Harlan Teklad, Madison, WI, USA). All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

Quantification of *ERα* deletion

To evaluate the efficiency of Cre-mediated *ERα* deletion in B cells, CD19^{+} cells were isolated from the bone marrow and spleen of 2-6 month old (NZB x NZW)F1 CD19$^{Cre/+}$; ERα$^{fl/-}$, CD19$^{Cre/+}$; ERα$^{fl/+}$, CD19$^{+/+}$; ERα$^{fl/+}$, and CD19$^{+/+}$; ERα$^{fl/-}$ mice. Briefly, bone marrow and spleen cells were isolated, passed through a 70 µm cell strainer to create a single cell suspension, and red blood cells lysed with ACK lysis buffer (Gibco, Waltham, MA, USA). Cells were labeled using CD19 Microbeads, and CD19^{+} cells were isolated using an AUTOMacsPro as per manufacturer protocol (Miltenyi Biotech, Bergisch-Gladbach, Germany). After isolation, DNA was isolated from CD19^{+} cells using a DNeasy Kit as per manufacturer protocol for cultured cells (Qiagen, Hilden, Germany).

Quantitative PCR was performed on isolated $CD19^+$ cell DNA as previously described (Nelson, 2016). Briefly, a primer set was developed flanking the LoxP sites around ERα Exon 3 (ERαDel F: 5'-TGGAATGAGACTTGTCTATCTTCG-3', ERαDel R: 5'-AACCAAGGAGAACAGAGAGACT-3'). From these primers, wild type and floxed *ERα* alleles flank regions of 699 and 773 bp, respectively, while *ERα* which has undergone recombination produces a product of 161 bp. In this QPCR assay, the wild type and floxed *ERα* alleles do not form products and only *ERα* that has undergone deletion is measured by this assay. A primer set in the unaffected *ERα* Exon 5 serves as a control in a separate reaction (ERαEx5 F: 5'-GGAAGGCCGAAATGAAATGGG-3' and ERαEx5 R: 5'-CCAACAAGGCACTGACCATC-3').

Survival studies

Survival of animals was monitored over one year. Animals were euthanized by CO_2 asphyxiation when they exhibited persistent albuminuria of 4+ (>2000 mg/dl) or physical signs of distress. Albuminuria was initially measured monthly, and with increasing frequency after a positive measurement using Albustix (Bayer Corporation, Elkhorn, IN, USA).

Histological analysis

Kidneys were collected from mice upon death or sacrifice at the end of the study. Tissues were fixed in 10% formalin for 96 hours, paraffin embedded, and sectioned. Kidney sections were stained with Periodic Acid Schiff (Sigma-Aldrich, St. Louis, MO, USA) and 100 glomeruli per kidney were evaluated as previously described (Yuan, 2013). Kidney sections were stained with anti-mouse IgG to detect immune complexes and color developed with the DAB kit (Vector Laboratories, Burlingame, CA, USA). To quantify the amount of immune complex (IC) staining in glomeruli, the color density of ≤20 immune complex stained and unstained glomeruli was measured and averaged. A

ratio of the two densities was calculated, and a higher number indicates more IC staining.

Serological analysis

Serum was collected from mice at monthly intervals beginning at 2 months of age. Enzyme Linked Immunosorbent Assay (ELISA) was used to measure serum antibody levels. Anti-dsDNA IgG ELISAs were done using Immulon 2 HB plates (Thermo Fisher Scientific, Waltham, MA, USA) coated with excess calf thymus dsDNA (Rockland Immunochemicals Inc., Limerick, PA, USA). Diluted serum samples were incubated on the plate, followed by anti-mouse IgG HRP conjugate, TMB substrate, and Stop Solution (Alpha Diagnostic International, San Antonio, TX, USA) was added and plates read at 450 nm. Plates were washed with 0.05% Tween-20 in PBS.

Total IgM, IgG1, IgG2a, IgG2b, and IgG3 ELISAs were done using Immulon 2 HB plates (Thermo) coated with 5 ng/ml capture antibody (Southern Biotech, Birmingham, AL, USA) and incubated overnight at 4ºC. Briefly, wells were incubated in blocking solution, incubated with samples and standards, then incubated with isotype specific alkaline phosphatate-labeled antibody, after which 1 mg/ml PNPP solution was applied for 20 minutes (Southern Biotech). Plates were read on a microplate reader at 415 nm. The concentrations of IgG1, IgG2a, IgG2b, and IgG3 were added together to find total IgG.

Anti-dsDNA Ig2a and IgG2b ELISAs were performed by applying standards and samples to dsDNA-coated Immulon 2 HB plates (Thermo Fisher Scientific). Isotype specific alkaline phosphatate-labeled antibodies, and PNPP solution (Southern Biotech) were added and plates read as for total isotype ELISAs.

Flow cytometry

To assess B cell development by flow cytometry, single cell suspensions were prepared and incubated with primary antibody for 30 minutes on ice. After staining for surface proteins, cells were incubated with Propidium Iodide (BD Biosciences, San Jose, CA, USA) for 10 minutes as a live/dead stain. After staining, cells were fixed with 0.6% formalin. The antibodies used were CD4-PE, CD5-PE, CD19-FITC, CD69-FITC, CD86-PE, B220-APC, CD93-BB515, CD279-APC, CXCR5-PECy7 (all BD Biosciences), IgM-FITC (Southern Biotech), IgD-APC-Cy7 (Biolegend, San Diego, CA, USA), CD21-eFlour450, and CD23-PE-Cy7 (eBioscience Inc., San Diego, CA, USA). Apoptosis was analyzed with Teleford reagent. Flow cytometry was performed with a BD LSRII Flow Cytometer and analyzed with FACSDiva software (BD Biosciences, v.8.0).

Statistics

Statistical analysis was done using SPSS software (IBM, v. 22). P values of less than or equal to 0.05 are considered significant. Kaplan Meyer survival curves with log rank tests were used to evaluate survival, and student's t tests or ANOVA were used to analyze QPCR, ELISA, histology, and flow cytometry data.

Results

Production of Experimental Animals

(NZB x NZW)F1 mice are a commonly used model of lupus. Characteristics of this model include the presence of autoreactive B and T cells, the production of autoreactive antibodies, and the development of fatal glomerulonephritis. To explore the role of ERα in B cells in lupus, we used the cre-loxP system to create a mouse model with *ERα* deletion in B cells on a lupus-prone genetic background.

NZB.CD19$^{Cre/+}$ mice were produced by backcrossing the *CD19-Cre* knockin allele onto the NZB background using simple sequence length polymorphism (SSLP) marker assisted selection. The 111 SSLP markers used for this process and their genetic locations are listed in Table 4.1. By the N5 generation, an average of only 7% of SSLP markers indicated remaining heterozygosity, while 93% were homozygous for NZB. In addition to our SSLP analysis, 733 SNPs were analyzed using DNA from an N5 NZB which was estimated by our SSLP analysis to be 94% NZB. SNP analysis showed this N5 NZB was 96% homozygous for NZB (Figure 4.1). All major areas of heterozygosity had been identified by our SSLP genotyping, and no remaining areas of residual heterozygosity were found in known SLE susceptibility loci. At this point, *CD19-Cre* was determined to be extensively backcrossed onto the NZB background.

NZB.CD19$^{Cre/+}$ mice were crossed with NZB.ERα$^{+/-}$ mice to produce NZB.CD19$^{Cre/+}$; ERα$^{+/-}$ mice. NZB.CD19$^{Cre/+}$; ERα$^{+/-}$ females were crossed with NZW.ERα$^{fl/fl}$ males to produce (NZB x NZW)F1 CD19$^{Cre/+}$; ERα$^{fl/-}$ mice, which should have no functional *ERα* alleles in CD19^{+} B cells after cre-mediated recombination. Previous studies have shown that global *ERα*$^{+/-}$ has no impact on lupus (Bynote, 2008). Therefore, any differences

Table 4.1 **SSLP markers used for the production of NZB.CD19-Cre mice**

Marker (location in cM, Mb)		
D1Mit316 (2.46, 10.3)	D6Mit209 (32.56, 75.5)	D12Nds2 (62.22, 115.1)
D1Mit169 (9.9, 24.1)	D6Mit328 (52.62, 112.7)	D13Mit16 (7.26, 20.4)
D1Mit123 (17.67, 39.2)	D6Mit14 (77.64, 145.6)	D13Mit275 (14.5, 37.4)
D1Mit132 (39.51, 77.1)	D7Mit21 (1.91, 3.3)	D13Mit13 (30.06, 56.6)
D1Mit440 (44.98, 90.7)	D7Mit267 (17.09, 30.3)	D13Mit126 (45.05, 85.5)
D1Mit495 (55.79, 129.5)	D7Mit82 (32.76, 58.8)	D13Mit74 (56.92, 106.7)
D1Mit159 (69.03, 161.6)	D7Mit248 (ND, 63.8)	D13Mit151 (64.72, 116.3)
D1Mit111 (76.73, 170.9)	D7Mit323 (54.45, 108)	D14Mit126 (11.94, 22.7)
D1Mit426 (84.32, 182.3)	D7Mit98 (60.49, 122.1)	D14Mit60 (24.6, 47.7)
D1Mit209 (96.35, 193.3)	D7Mit358 (67.27, 129.9)	D14Mit5 (31.49, 60.3)
D2Mit1 (2.33, 3.8)	D7Mit101 (69.01, 132.8)	D14Mit68 (37.61, 72.9)
D2Mit83 (19.38, 28.8)	D7Mit332 (77.87, 141.2)	D14Mit106 (50.9, 100.6)
D2Mit156 (31.66, 56.9)	D8Mit155 (2.14, 5)	D14Mit177 (60.21, 116.8)
D2Mit327 (40.88, 69.3)	D8Mit289 (16.47, 29.9)	D15Mit252 (8.54, 22.6)
D2Mit94 (47.93, 80)	D8Mit69 (29.7, 59.2)	D15Mit143 (19.62, 52)
D2Mit395 (59.97, 119.4)	D8Mit178 (34.43, 73.6)	D15Mit67 (32.17, 70)
D2Mit411 (80.04, 159.4)	D8Mit211 (52, 105.2)	D15Mit107 (39.79, 84.2)
D2Mit145 (86.75, 166.2)	D8Mit49 (72.38, 126.6)	D15Mit161 (52.78, 96.8)
D3Mit203 (10.82, 26.8)	D9Mit90 (17.8, 32.3)	D16Mit131 (3.41, 7.3)
D3Mit51 (ND, 77.0)	D9Mit129 (24.45, 43.7)	D16Mit60 (23.27, 32.7)
D3Mit26 (34.97, 79.5)	D9Mit123 (40.88, 73.4)	D16Mit139 (37.28, 65.7)
D3Mit311 (40.14, 92.8)	D9Mit355 (51.41, 98.7)	D16Mit52 (53.73, 92.7)
D3Mit320 (66.75, 143.2)	D9Mit55 (65.28, 114.7)	D17Mit164 (2.11, 3.9)
D3Mit19 (ND, 164.4)	D10Mit213 (9.75, 20.1)	D17Mit51 (19.74, 43.6)
D4Mit193 (13.99, 32.3)	D10Mit20 (34.83, 66.5)	D17Mit10 (ND, 51.0)
D4Mit17 (33.96, 63)	D10Mit230 (45.28, 89.7)	D17Mit93 (45.2, 74.2)
D4Mit9 (43.34, 94.7)	D10Mit233 (61.58, 113.8)	D17Mit122 (52.25, 83.5)
D4Mit308 (57.66, 123.8)	D10Mit297 (72.31, 124.5)	D18Mit222 (8.08, 14.7)
D4Mit42 (82.64, 150.9)	D11Mit71 (4.7, 6.8)	D18Mit177 (21.39, 41.1)
D5Mit348 (11.97, 24.4)	D11Mit189 (27.39, 45.3)	D18Mit186 (45.63, 72.2)
D5Mit352 (18.4, 36)	D11Mit5 (40.59, 67)	D18Mit144 (57.79, 85.7)
D5Mit201 (39.55, 75.6)	D11Mit285 (54.64, 89.8)	D19Mit96 (15.54, 21.9)
D5Mit314 (53.25, 110.1)	D11Mit333 (71.83, 108.6)	D19Mit88 (32.23, 37.3)
D5Mit97 (76.1, 137.5)	D12Mit182 (5.52, 10.9)	D19Mit90 (35.97, 42.3)
D5Mit143 (89.8, 151.8)	D12Mit60 (15.54, 35.5)	D19Mit103 (48.46, 53.8)
D6Mit138 (1.81, 4.5)	D12Mit91 (30.06, 72.8)	
D6Mit116 (11.5, 21.1)	D12Mit158 (38.14, 83.7)	
D6Mit123 (27.76, 56.9)	D12Mit7 (ND, 103)	

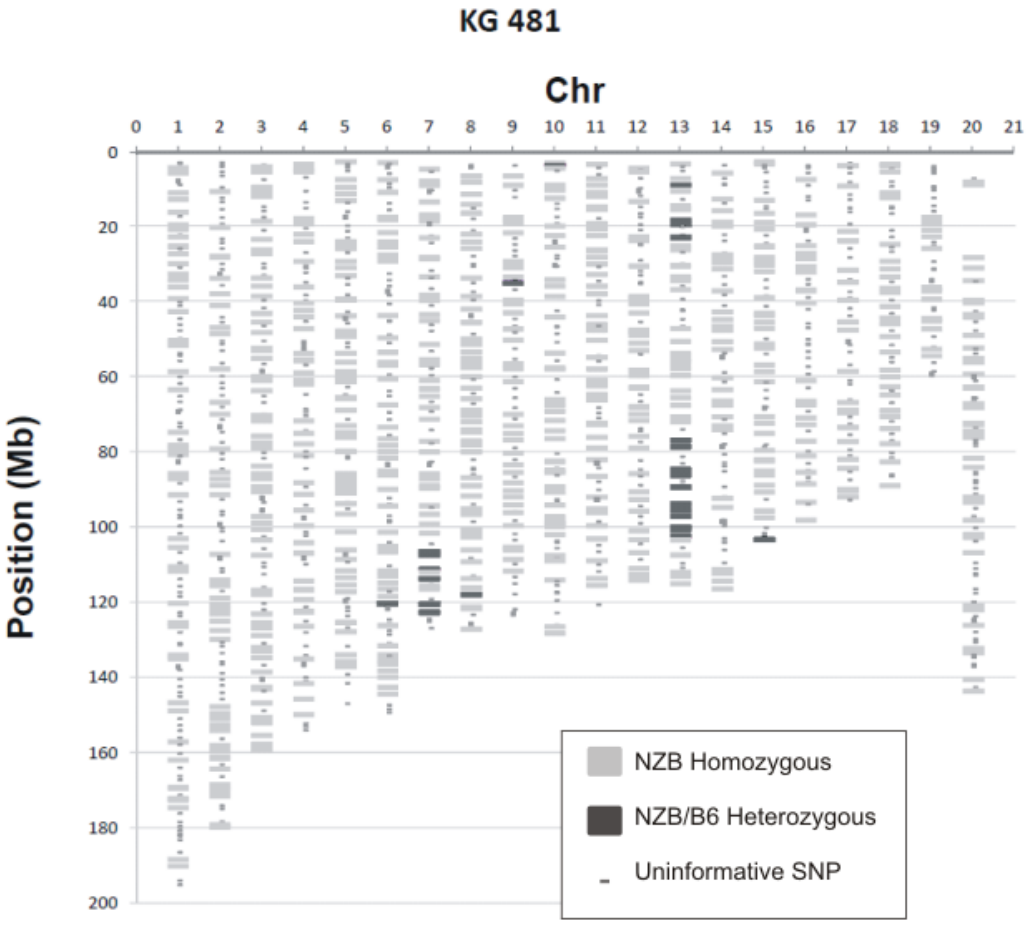

Figure 4.1 **The *CD19-Cre* knockin allele was backcrossed onto the NZB genetic background**

Figure 4.1 **The *CD19-Cre* knockin allele was backcrossed onto the NZB genetic background**

SNP analysis was performed on an NZB.CD19-Cre N5 female mouse to determine the purity of the genetic background. 96% of informative SNPs were NZB homozygous, while 4% of SNPs showed residual B6/NZB heterozygosity, including the region surrounding the *CD19-Cre* knockin allele on distal chromosome 7.

observed in lupus in (NZB x NZW)F1 $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice are due to the deletion of *ERα* in B cells.

On the (NZB x NZW)F1 Genetic Background, the CD19-Cre Knockin Allele Causes Only Moderately Efficient Deletion of ERα and Increases B cell Apoptosis

The *CD19-Cre* knockin allele was previously shown to have a deletion efficiency of 75-80% in bone marrow pre-B cells and 90-95% in splenic B cells on the 129 genetic background (Rickert, 1997). To assess the efficiency of *CD19-Cre* mediated deletion of *ERα* on the (NZB x NZW)F1 genetic background, $CD19^{+}$ cells from bone marrow and spleen were isolated from 2-6 month old mice, and DNA from these cells was analyzed for deletion efficiency. The efficiency of deletion was not different at different ages, nor were there any sex-related differences in deletion efficiency. No deletion was detected in any $CD19^{+/+}$; $ER\alpha^{fl/+}$ or $CD19^{+/+}$; $ER\alpha^{fl/-}$ samples (not shown). $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice have an average of 27% deletion efficiency in $CD19^{+}$ bone marrow cells, which is not significantly different from $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice which have an average deletion efficiency of 18% (Figure 4.2a). $CD19^{+}$ spleen cells from $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice have an average deletion efficiency of 53%, while $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice have an average deletion efficiency of 54%. Our results affirm previous findings which showed that that the efficiency of *CD19-Cre* mediated gene deletion is higher in splenic B cells than bone marrow B cells; however, deletion efficiency on the (NZB x NZW)F1 genetic background is significantly lower than has been previously reported.

We posit that the lupus-prone genetic background of (NZB x NZW)F1 mice is the reason only a moderate level of *ERα* deletion is observed. Cre recombinase can cleave at loxP-like sites throughout the mammalian genome, causing dsDNA breaks (Loonstra, 2001). Many lupus patients cannot effectively repair dsDNA breaks (Bassi, 2008; Davies, 2012). Unrepaired dsDNA breaks lead to cell death. This means that cells that express

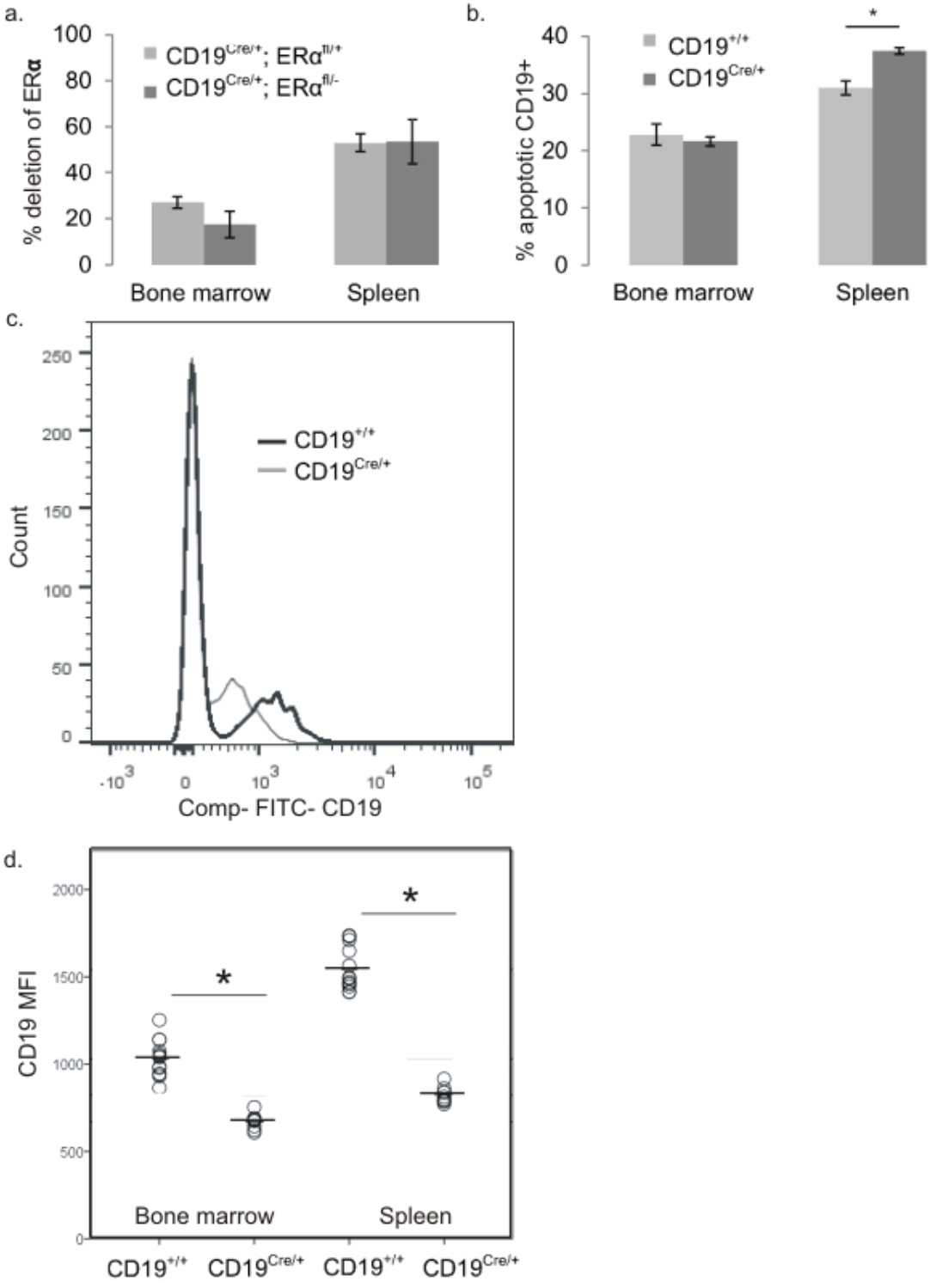

Figure 4.2 **The *CD19-Cre* knockin allele causes apoptosis and decreased *CD19* expression in (NZB x NZW)F1 mice**

Figure 4.2 **The *CD19-Cre* knockin allele causes apoptosis and decreased *CD19* expression in (NZB x NZW)F1 mice**

The efficiency of *ERα* deletion was measured in (NZB x NZW)F1 mice. (a) *ERα* deletion in $CD19^+$ bone marrow and spleen cells was moderately successful. (b) The rate of apoptosis in $CD19^+$ splenocytes was increased in female mice with the *CD19-Cre* knockin allele (p=0.003). Fluorescent intensity of CD19 staining on B cells was measured in mice with and without the *CD19-Cre* knockin allele. (c) CD19 expression was reduced in mice with the *CD19-Cre* knockin allele (representative sample shown). (d) Mean fluorescent intensity of CD19 staining was consistently and significantly reduced in both bone marrow and spleen B cells ($p=6.0x10^{-9}$, $p=3.3x10^{-13}$).

low levels of cre recombinase may have a survival advantage, and this could contribute to the decreased efficiency of cre-mediated deletion observed in (NZB x NZW)F1 mice. Our lab has previously observed inefficient *Lck-Cre* mediated deletion of *ERα* in T cells in (NZB x NZW)F1 mice, as well as an increase in T cell apoptosis (Nelson, 2016). We examined apoptosis in young (NZB x NZW)F1 mice and found that females that have the *CD19-Cre* knockin allele had significantly more apoptotic $CD19^{+}$ splenocytes than $CD19^{+/+}$ mice (Figure 4.2b). No difference was observed in apoptosis in $CD19^{+}$ bone marrow cells.

In our (NZB x NZW)F1 model, deletion of *ERα* in B cells was only moderately efficient, and the B cell population in $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice was composed of a mixture of $ER\alpha^{+/-}$ and $ER\alpha^{-/-}$ cells. The mixed nature of the B cell compartment in this model impacted the magnitude of the effects of B cell ERα on lupus that could be observed.

The CD19-Cre Knockin Allele Causes Reduced CD19 Expression

The *CD19-Cre* knockin allele by itself has the potential to impact the lupus phenotype in (NZB x NZW)F1 mice. Mice that carry the *CD19-Cre* knockin allele have significantly less *CD19* expression on B cells compared to $CD19^{+/+}$ littermates (Figure 4.2c). In the bone marrow, *CD19-Cre* mice have an average of 65% as much CD19 as $CD19^{+/+}$ mice, and in the spleen they have an average of 53% as much CD19 (Figure 4.2d). This decrease in *CD19* expression could impact the strength of B cell receptor signaling and alter the negative selection of autoreactive B cells. The *CD19-Cre* knockin allele could also affect the lupus phenotype through the toxic off-target effects of cre expression. Debris from apoptotic B cells is a source of autoantigen, and leads to acceleration of disease (Trebeden-Negre, 2003). Because of its potential to impact lupus, the effects of the *CD19-Cre* knockin allele alone were examined in (NZB x NZW)F1 mice.

The CD19-Cre Knockin Allele Causes Accelerated Mortality on the (NZB x NZW)F1 Genetic Background

Comparison of $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ and $CD19^{+/+}$; $ER\alpha^{fl/+}$ (NZB x NZW)F1 mice revealed that the *CD19-Cre* knockin allele itself caused accelerated mortality in female and male mice. Female $CD19^{+/+}$; $ER\alpha^{fl/+}$ mice had a median survival time of 211 days, while $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ females had a median survival time of 171 days (Figure 4.3a). The median lifespan of (NZB x NZW)F1 $CD19^{+/+}$; $ER\alpha^{fl/+}$ females is similar to the median survival of (NZB x NZW)F1.$ER\alpha^{+/+}$ females which was 238 days (Bynote, 2008). Similarly, male $CD19^{+/+}$; $ER\alpha^{fl/+}$ mice had a median survival time of 301 days and $CD19^{Cre/+}$; $ER\alpha^{fl/+}$males had a median survival time of 257 days (Figure 4.3b). The median survival time of (NZB x NZW)F1 $CD19^{+/+}$; $ER\alpha^{fl/+}$ males of 301 days is similar to the median survival of (NZB x NZW)F1.$ER\alpha^{+/+}$ males which was 321 days (Bynote, 2008). Although we are unable to distinguish whether it is an effect of cre recombinase or *CD19* heterozygosity, it is clear that the *CD19-Cre* knockin allele causes significantly accelerated mortality in (NZB x NZW)F1 mice. Therefore, the appropriate control group for mice with B cell specific deletion of *ERα* is mice with the *CD19-Cre* knockin allele.

B cell Specific Deletion of ERα Extends Survival

Our lab has previously shown that total body *ERα* knockout significantly attenuates the development of lupus and extends survival in (NZB x NZW)F1 female and male mice (Bynote, 2008). To determine the effects of B cell specific ERα deletion on survival, mice were monitored for up to one year. Female $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ control mice had a median survival of 171 days, which is significantly shorter than $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice that had a median survival time of 239 days (Figure 4.3c). 25% of $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ females and 2% of $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ female controls survived until the end of the study.

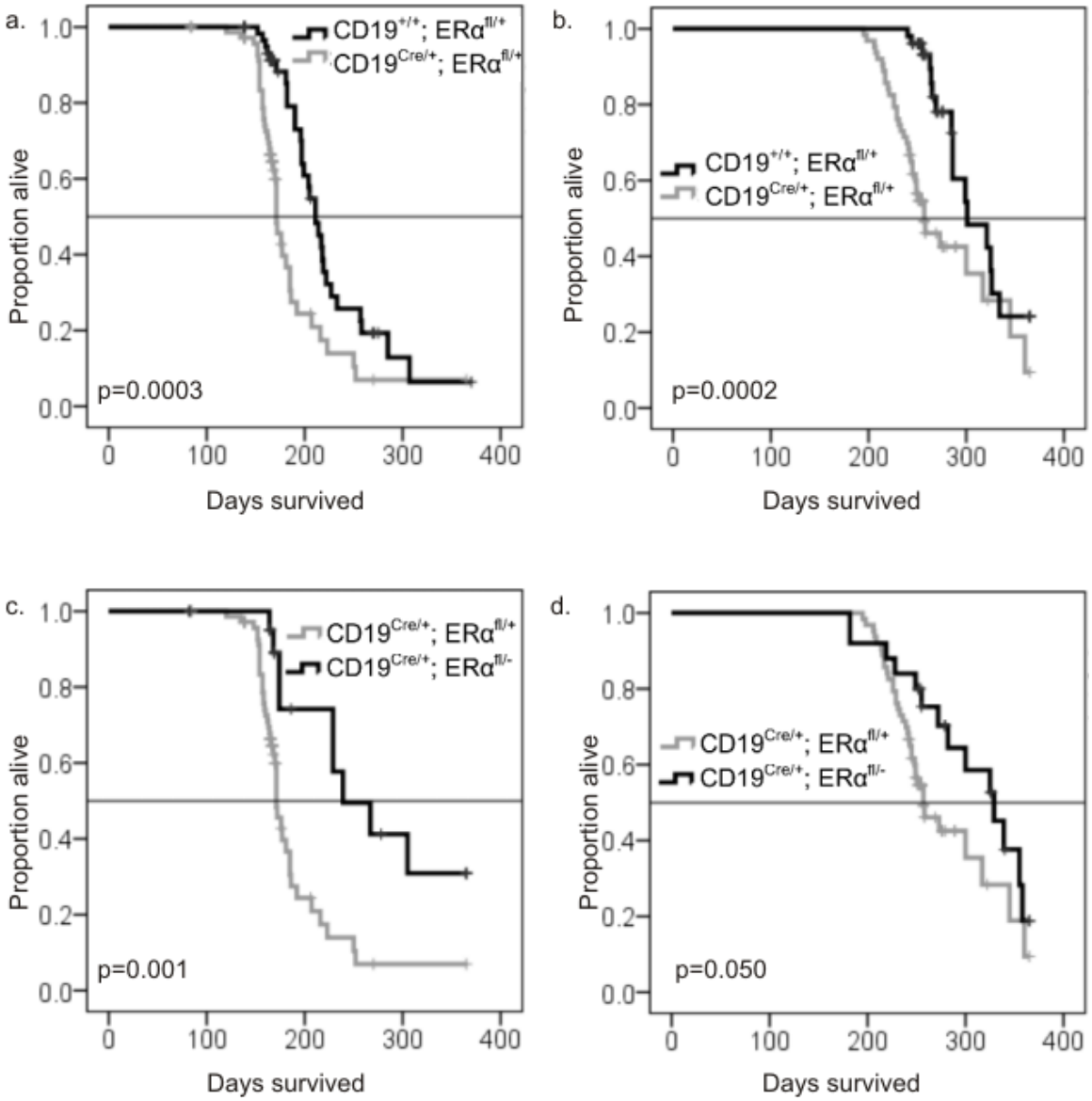

Figure 4.3 **Both the *CD19-Cre* knockin allele and B cell *ERα* significantly impact survival**

Figure 4.3 **Both the *CD19-Cre* knockin allele and B cell *ERα* significantly impact survival**

Survival was monitored for up to one year. (a) Female and (b) male $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice have significantly shorter median survival compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$ mice (p=0.0003 females and p=0.0002 males). Compared to $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ control mice, $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ (c) female and (d) male mice have significantly longer median survival (p=0.001 females and p=0.050 males).

Although significant, the 68 day increase in median survival observed in female mice with *ERα* deletion in B cells is less dramatic than the survival difference observed in female mice with *ERα*$^{-/-}$, where more than half of the population survived for 18 months (Bynote, 2008). This difference is likely due, at least in part, to the large proportion of B cells in CD19$^{Cre/+}$; ERα$^{fl/-}$ mice that retain *ERα* expression. Additionally, ERα signaling in other cell types may also contribute to lupus in (NZB x NZW)F1 mice.

Male mice with B cell specific *ERα* deletion also experience significantly delayed mortality. Male CD19$^{Cre/+}$; ERα$^{fl/+}$ control mice had a median lifespan of 257 days, which is significantly shorter that of CD19$^{Cre/+}$; ERα$^{fl/-}$ males which had a median lifespan of 329 days (Figure 4.3d). Only 3% of male CD19$^{Cre/+}$; ERα$^{fl/+}$ mice survived until the end of the study, and 12.5% of CD19$^{Cre/+}$; ERα$^{fl/-}$ mice survived for one year. The 72 day difference in the median survival of male mice in this study is only slightly less than the 85-113 day difference in median survival observed between *ERα*$^{+/+}$ or *ERα*$^{+/-}$ and *ERα*$^{-/-}$ male (NZB x NZW)F1 mice (Bynote, 2008).

Mice from All Groups Develop Glomerulonephritis

(NZB x NZW)F1 mice typically develop and succumb to severe glomerulonephritis. Kidneys were collected from mice when they showed signs of advanced illness or reached one year of age, and were removed from the survival study. Periodic Acid Schiff stained sections were analyzed to determine the extent of glomerular damage. (NZB x NZW)F1 mice with the *CD19-Cre* knockin allele developed glomerulonephritis that was histologically indistinguishable from *CD19*$^{+/+}$ mice (data not shown). Almost all female and male CD19$^{Cre/+}$; ERα$^{fl/+}$ and CD19$^{Cre/+}$; ERα$^{fl/-}$ mice had ≥ 50% abnormal glomeruli, which indicates severe glomerulonephritis (Figure 4.4a). The B cell *ERα* genotype had no impact on the extent of glomerulonephritis. Representative PAS stained images show a similar extent of damage in both CD19$^{Cre/+}$; ERα$^{fl/+}$ and CD19$^{Cre/+}$; ERα$^{fl/-}$ mice (Figure

4.4b). Additionally, no difference was found in the amount of immune complex deposition in the glomeruli (Figure 4.4c). Therefore, although mice with B cell specific deletion of *ERα* survive significantly longer than control mice, both groups of mice develop severe glomerulonephritis. This also indicates that the *CD19-Cre* knockin allele does not cause mortality that is not related to glomerulonephritis.

Mice with B cell Specific ERα Deletion Produce Fewer Pathogenic Autoantibodies

The production of high levels of dsDNA-reactive antibodies is characteristic of lupus. In lupus patients and (NZB x NZW)F1 mice, serum levels of dsDNA IgG antibodies correlate with disease severity. The development of autoreactive B cells and production of pathogenic autoantibodies are promoted by ERα signaling (Hill, 2011). To determine the effect of *ERα* expression in B cells on autoantibody production, serum anti-dsDNA IgG levels were measured by ELISA. Serum levels of anti-dsDNA IgG were significantly higher in control $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice at 5 months of age compared to female $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice (Figure 4.5a). Similarly, male $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ control mice had significantly higher levels of anti-dsDNA IgG antibodies compared to $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ males (Figure 4.5b). Lower levels of pathogenic autoantibodies in mice with B cell specific *ERα* deletion indicate amelioration of lupus, and are consistent with the attenuated mortality observed in these mice.

Different isotypes of anti-dsDNA IgG antibodies have different pathogenic potentials; autoreactive IgG2a and IgG2b antibodies are especially pathogenic because they activate both complement and Fcγ receptors (Azeredo da Silveira, 2002). To determine if these antibodies were affected by *ERα* in B cells, serum levels of anti-dsDNA IgG2a and anti-dsDNA IgG2b were measured by ELISA at 4 months of age in females and 7 months of age in males. Compared to $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ controls, levels of anti-dsDNA

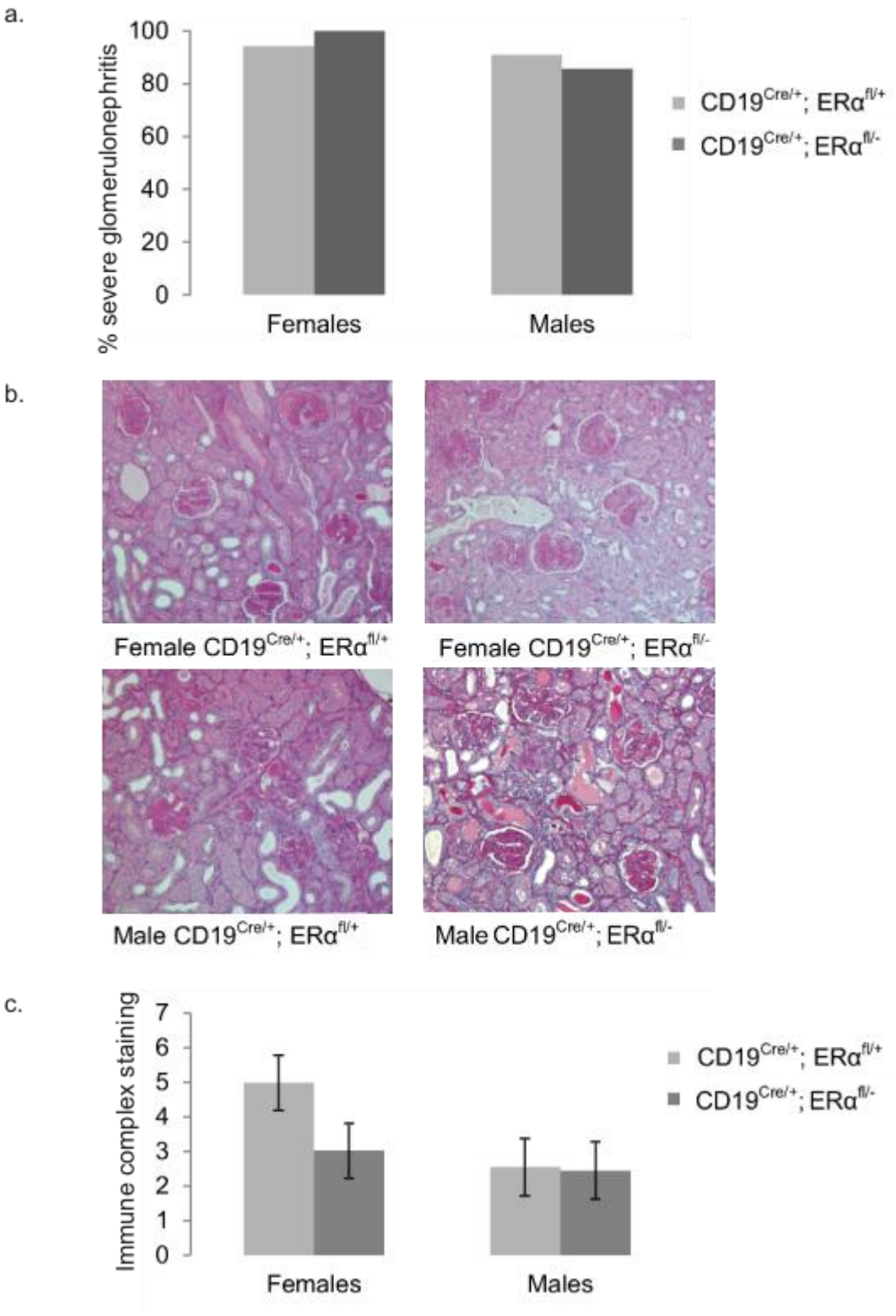

Figure 4.4 **B cell *ERα* deletion does not prevent the development of glomerulonephritis**

Figure 4.4 **B cell *ERα* deletion does not prevent the development of glomerulonephritis**

(a) The majority of mice of both sexes developed severe glomerulonephritis by the end of the survival study. (b) Representative histological sections show abnormalities characteristic of glomerulonephritis. (c) Semi-quantitative analysis of immune complex staining showed immune complex deposition occurred in equally in $CD19^{Cre/+}$; $ERα^{fl/-}$ and $CD19^{Cre/+}$; $ERα^{fl/+}$ mice of both sexes.

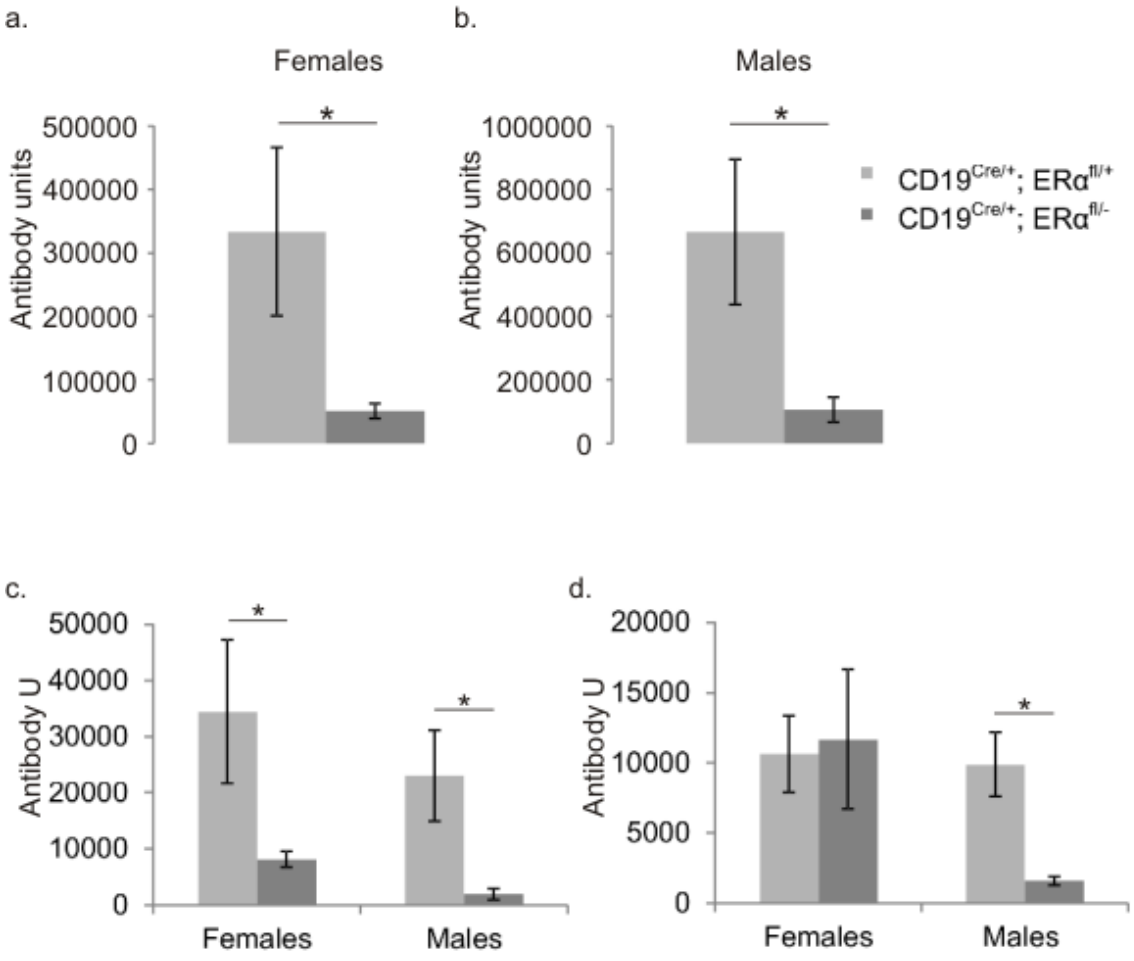

Figure 4.5 part 1 **B cell specific *ERα* deletion causes reduced production of pathogenic autoantibodies but does not affect total antibody production**

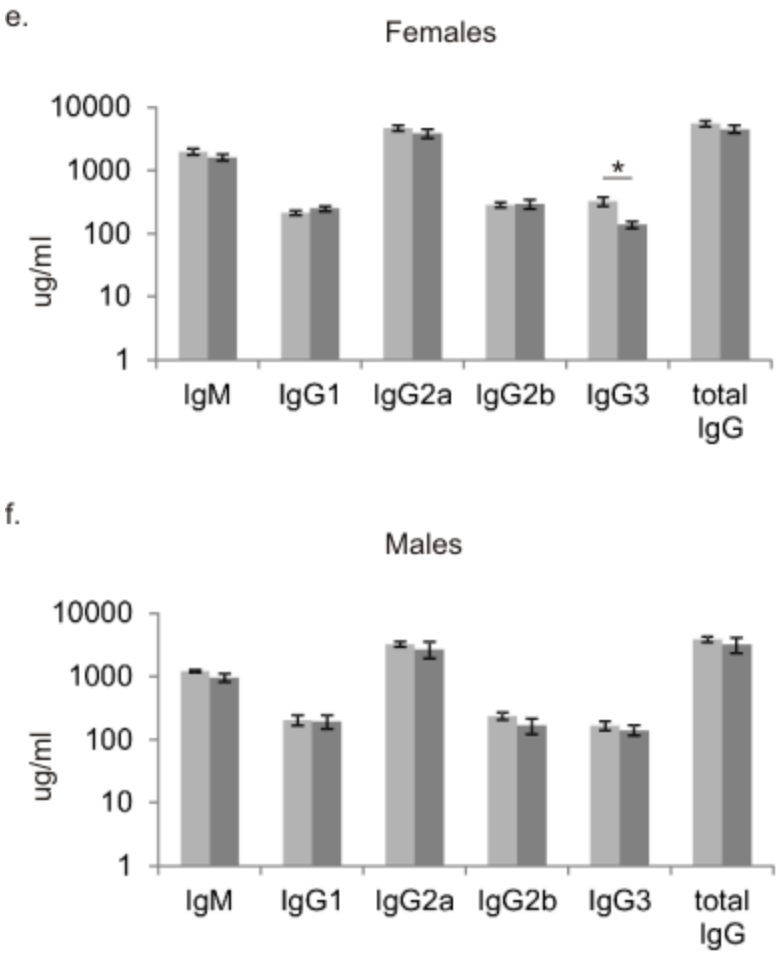

Figure 4.5 part 2 **B cell specific *ERα* deletion causes reduced production of pathogenic autoantibodies but does not affect total antibody production**

Figure 4.5 **B cell specific *ERα* deletion causes reduced production of pathogenic autoantibodies but does not affect total antibody production**

(a) Female and (b) male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice produce less α-dsDNA IgG than $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ controls (a. p=0.03; b. p=0.02). (c) Less α-dsDNA IgG2a is produced by both female and male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice (p=0.04 females and p=0.02 males). (d) Male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice produce less α-dsDNA IgG2b (p=0.002). There is no difference in total IgM, total IgG, or IgG1, IgG2a, or IgG2b in (e) female and (f) male mice. Female $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice produce less total IgG3 than $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ controls (p=0.005).

The data for 4.5e-f was produced by Kimberly Bynote and analyzed by Dana Tabor.

IgG2a were significantly lower in both female and male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice (Figure 4.5c). Additionally, compared to $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ controls, male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice had lower serum levels of anti-dsDNA IgG2b antibodies, but no difference was observed in anti-dsDNA IgG2b levels in female mice (Figure 4.5d).

To evaluate the effect of B cell specific *ERα* deletion on total antibody production, and to determine if the significant reduction in anti-dsDNA IgG autoantibodies is due to global changes in antibody production, we measured serum IgM, IgG1, IgG2a, IgG2b, and IgG3 levels by ELISA. Total IgG was calculated by totaling the antibodies of the various IgG isotypes. In female mice, the only difference detected was that $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice produce significantly more IgG3 than $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ females (Figure 4.5e). In male mice, there were no differences in the production of IgM or any IgG isotype (Figure 4.5f). Therefore, the reduction in dsDNA IgG is not due to a global decrease in antibody production, but is specific to dsDNA antibodies. Thus, it is likely that the dramatic reduction in anti-dsDNA IgG antibodies is primarily responsible for the attenuation of lupus in mice with B cell specific deletion of *ERα*.

B cell ERα Deletion Leads to Decreased B cell Activation

ERα affects the development of B cells in the bone marrow. Specifically, E2 causes a decrease in developing bone marrow B cells on a mixed C57BL/6/129 genetic background (Thurmond, 2000; Erlandsson, 2003). Reports vary on the effect of *ERα* knockout on B cell populations in the bone marrow; however there is consensus that the effects of E2 on B cell populations are mainly mediated by signaling through ERα (Thurmond, 2000; Erlandsson, 2003). It is not known if these effects are B cell intrinsic.

To evaluate the effects of B cell intrinsic ERα on B cell populations in the bone marrow, cells from 3 month old pre-autoimmune (NZB x NZW)F1 mice were analyzed by flow

cytometry. In females, but not males, we observed a decrease in the percentage of pre-B cells and increase in the percentage of immature B cells, which was due to the *CD19-Cre* knockin allele (Table 4.2). The deletion of *ERα* from B cells did not cause changes in the bone marrow or spleen populations in either female or male mice.

The effect of global *ERα* knockout on B cell populations in the spleen has not previously been reported. In order to have a comparison for our mice with B cell specific *ERα* deletion, we evaluated follicular and marginal zone B cell populations in 3 month old $ERα^{-/-}$ and $ERα^{+/+}$ (NZB x NZW)F1s. The population of follicular B cells was not affected by *ERα* genotype (Table 4.3). However, the population of marginal zone B cells was significantly increased in $ERα^{-/-}$ female mice. This was somewhat unexpected because the marginal zone B cell population is enriched for autoreactive B cells compared to follicular B cells, and $ERα^{-/-}$ female mice produce less autoantibodies than $ERα^{+/+}$ mice (Grimaldi, 2001; Bynote, 2008). However, the marginal zone B cell population also increases significantly in high-estrogen conditions or upon treatment with an ERα specific agonist (Grimaldi, 2006; Hill, 2011). Female $ERα^{-/-}$ mice have significantly elevated serum E2 (Couse, 1995), which may be the reason for the increase in marginal zone B cells in these mice.

The same analysis of spleen populations was done on 3 month old (NZB x NZW)F1 *CD19-Cre* mice. The percentage of follicular and marginal zone B cells is similar between $ERα^{+/+}$ and $CD19^{+/+}$; $ERα^{fl/+}$ mice. *ERα* deletion in B cells did not have a significant impact on follicular or marginal zone B cell populations, but the *CD19-Cre* knockin allele significantly impacted these populations (Tale 4.4). The *CD19-Cre* knockin allele causes an increase in the proportion of follicular B cells and a decrease in the proportion of marginal zone B cells. Several other lymphocyte populations were examined in the spleen, and no significant differences were caused by B cell *ERα*

Table 4.2 **Bone marrow B cell populations in (NZB x NZW)F1 mice**

	$CD19^{Cre/+}$; $ER\alpha^{fl/-}$	**$CD19^{Cre/+}$; $ER\alpha^{fl/+}$**	**$CD19^{+/+}$; $ER\alpha^{fl/+}$**
Females			
Pre-B cells (IgM^{-} IgD^{-})	40.6 ± 1.9 *	42.6 ± 2.8 *	53.0 ± 1.5
Immature B cells (IgM^{+} IgD^{-})	27.7 ± 1.4 *	31.9 ± 1.6 *	18.4 ± 1.6
Recirculating B cells ($IgM^{+/-}$ IgD^{+})	29.3 ± 1.2 †	23.2 ± 1.8	26.6 ± 1.2
Males			
Pre-B cells (IgM^{-} IgD^{-})	50.1 ± 5.7	48.9 ± 3.1	50.9 ± 5.3
Immature B cells (IgM^{+} IgD^{-})	24.5 ± 3.0	24.4 ± 1.8	25.3 ± 2.9
Recirculating B cells ($IgM^{+/-}$ IgD^{+})	24.0 ± 4.3	24.9 ± 2.5	21.8 ± 3.2

% of live B220+ cells

* Indicates $p \leq 0.05$ compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$
† indicates $p \leq 0.05$ compared to $CD19^{Cre/+}$; $ER\alpha^{fl/+}$

3 month old mice were used for this experiment.

Table 4.3 **Spleen B cell populations in (NZB x NZW)F1 mice**

	***ERα*$^{+/+}$**	***ERα*$^{-/-}$**
Females		
Follicular B cells ($CD21^+$ $CD23^+$)	55.4 ± 1.4	54.5 ± 0.9
Marginal zone B cells ($CD21^+$ $CD23^-$)	26.6 ± 1.1	36.4 ± 0.9 *
Males		
Follicular B cells ($CD21^+$ $CD23^+$)	57.2 ± 2.0	53.9 ± 5.3
Marginal zone B cells ($CD21^+$ $CD23^-$)	28.6 ± 2.1	31.5 ± 2.2

% of live $B220^+$ $CD5^-$ cells
* indicates $p \leq 0.05$ compared to $ER\alpha^{+/+}$

3 month old mice were used for this experiment.

deletion or *CD19-Cre* in transitional B cell, or T follicular helper cell populations (Table 4.4). These results suggest that *CD19-Cre* reduces the relative abundance of marginal zone B cells in the spleen, but that deletion of *ERα* in B cells does not affect the relative abundance of splenic B cell populations. Thus, the attenuated development of lupus in $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice is not the result of a shift in the relative abundance of splenic B cell subsets

Previous studies from our lab suggest that ERα may promote lupus by enhancing immune cell activation (Yoachim, 2015). Therefore, the percentage of activated B and T cells in the spleen was examined in these young mice. Female $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice had significantly fewer activated B cells than $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice (Table 4.5). This was not observed in males at this age. There was no change in proportion of activated T cells in mice of either sex. This suggests that ERα promotes B cell activation in female lupus-prone mice in a B cell intrinsic manner, and further suggests that the ability of ERα to promote B cell activation may underlie the ability of ERα to promote lupus.

Table 4.4 **Spleen lymphocyte populations in (NZB x NZW)F1 mice**

	$CD19^{Cre/+}$; $ER\alpha^{fl/-}$	**$CD19^{Cre/+}$; $ER\alpha^{fl/+}$**	**$CD19^{+/+}$; $ER\alpha^{fl/+}$**
Females			
Follicular B cells ($CD21^+$ $CD23^+$)	66.0 ± 1.0 *	68.1 ± 1.6 *	59.8 ± 1.2
Marginal zone B cells ($CD21^+$ $CD23^-$)	18.9 ± 1.6 ^	18.0 ± 1.1 *	25.2 ± 2.0
Transitional B cells ($AA4.1^+$)	5.6 ± 0.6	6.0 ± 0.5	5.6 ± 0.5
T follicular helper cells (% of live $CD4^+$ $CXCR5^+$ $PD1^+$)	51.9 ± 3.9	49.3 ±2.4	46.0 ± 1.4
Males			
Follicular B cells ($CD21^+$ $CD23^+$)	67.7 ± 1.6 *	65.0 ± 1.3 ^	60.4 ± 1.9
Marginal zone B cells ($CD21^+$ $CD23^-$)	20.8 ± 0.3 *	19.2 ± 0.4 *	25.0 ± 1.4
Transitional B cells ($AA4.1^+$)	4.2 ± 0.3	5.3 ± 0.5	4.8 ± 0.3
T follicular helper cells (% of live $CD4^+$ $CXCR5^+$ $PD1^+$)	45.8 ± 2.8	46.9 ± 1.5	42.5 ± 1.3

B cells- % of live $B220^+$ $CD5^-$ cells
* indicates $p \leq 0.05$ compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$
^ indicates $p \leq 0.1$ compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$

3 month old mice were used for this experiment.

Table 4.5 **B and T cell activation in (NZB x NZW)F1 mice**

	$CD19^{Cre/+}$; $ER\alpha^{fl/-}$	**$CD19^{Cre/+}$; $ER\alpha^{fl/+}$**	**$CD19^{+/+}$; $ER\alpha^{fl/+}$**
Females			
Activated B cells (% live of $B220^{+}$ $CD86^{+}$)	1.9 ± 0.3 †	5.7 ± 0.8 *	3.0 ± 0.6
Activated T cells (% live of $CD4^{+}$ $CD69^{+}$)	5.5 ± 0.9	9.2 ± 2.3	6.2 ± 0.8
Males			
Activated B cells (% live of $B220^{+}$ $CD86^{+}$)	2.3 ± 0.4 ^	2.7 ± 0.4	3.5 ± 0.5
Activated T cells (% live of $CD4^{+}$ $CD69^{+}$)	4.7 ± 0.6	5.2 ± 0.9	5.4 ± 0.8

* Indicates $p \leq 0.05$ compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$
^ indicates $p \leq 0.1$ compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$
† indicates $p \leq 0.05$ compared to $CD19^{Cre/+}$; $ER\alpha^{fl/+}$

3 month old mice were used for this experiment.

Discussion

To study the impact of ERα in B cells on lupus, we created (NZB x NZW)F1 mice with *ERα* deletion in B cells. This was accomplished using the *CD19-Cre* knockin allele which causes the expression of cre recombinase in B cells, and an allele of *ERα* flanked by loxP sites (floxed *ERα*). The cre-loxP system is widely used in mammalian systems to cause targeted deletion of DNA segments from the genome. Although this system is potentially very useful, models created with the cre-loxP system are often under-characterized. In our studies, we found that the *CD19-Cre* knockin allele by itself caused significant effects in (NZB x NZW)F1 mice. Cre recombinase can cleave pseudo-loxP sites throughout the mammalian genome, causing severe DNA damage (Thyagarajan, 2000). Indeed, we observed a small increase in apoptotic $CD19^{+}$ splenocytes in *CD19-Cre* mice.

The *CD19-Cre* knockin allele not only causes cre recombinase expression in B cells, but because it is a knockin allele, it interrupts the coding region of *CD19,* resulting in a null allele. To our knowledge, the effect of *CD19* heterozygosity on B cell populations has not been reported. However, it is known that total *CD19* knockout in (NZB x NZW)F1 mice accelerates the development of lupus (Watanabe, 2010). In our (NZB x NZW)F1 *CD19-Cre* mice we were unable to determine whether the effects of the *CD19-Cre* allele were due to the decreased expression of *CD19* or the expression of cre recombinase, both of which could contribute to the changes we observed.

Survival studies showed that female and male $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice (which carry the *CD19-Cre* knockin allele but do not have B cell *ERα* deletion) have a significantly reduced median survival compared to $CD19^{+/+}$; $ER\alpha^{fl/+}$ mice. In females, the *CD19-Cre* knockin allele also caused changes in B cell populations in the bone marrow, and differences in B cell populations were observed in both sexes in the spleen. Therefore, in

the studies presented here, $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice are the appropriate controls for $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice with B cell specific *ERα* deletion. A recent study from our lab showed that the *Lck-Cre* allele significantly accelerates lupus in (NZB x NZW)F1 mice by causing increased apoptosis and an increase in the proportion of activated T cells (Nelson, 2016). Although we did find that the *CD19-Cre* knockin allele caused increased apoptosis of $CD19^{+}$ splenocytes, it did not lead to an increase in B or T cell activation. Although the effects of cre were not identical, these studies show that cre-loxP systems can have significant, unintended effects in some models.

In addition to examining the effects of the *CD19-Cre* knockin allele on survival and B cell populations, the efficiency of *ERα* deletion in $CD19^{+}$ B cells from $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ and $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice was analyzed. On the 129 genetic background, *CD19-Cre* causes 75-80% deletion in bone marrow B cells and 90-95% deletion in spleen B cells (Rickert, 1997). The deletion efficiency was much lower in (NZB x NZW)F1 *CD19-Cre* mice. Only ~20% of $CD19^{+}$ bone marrow cells had *ERα* deletion while ~50% of $CD19^{+}$ B cells had *ERα* deletion. These results indicate that the B cells in $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice are a mixed population of $ERα^{+/-}$ and $ERα^{-/-}$ cells. Therefore, the effects that we have observed in these studies are the result of loss of ERα signaling in only a portion of the B cell population.

Loss of ERα signaling in a portion of B cells in (NZB x NZW)F1 mice significantly attenuated lupus. Female and male $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice survived significantly longer than $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice. The increase in survival time observed in mice with B cell *ERα* deletion is not as dramatic as the increase in survival that occurred when *ERα* was knocked out globally in (NZB x NZW)F1 mice. However, we speculate that the increase in survival time between $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ and $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice would be even

greater if there was more complete deletion of *ERα* in B cells. It is also possible that ERα signaling in other cell types contributes to lupus.

Glomerulonephritis is a highly penetrant manifestation of lupus in (NZB x NZW)F1 mice and is the main cause of death. At the end of the survival study, kidneys were collected and analyzed for glomerulonephritis and immune complex deposition. Almost all mice from each group had severe glomerulonephritis. There were no significant differences in the proportion of mice with severe glomerulonephritis or in the amount of immune complex deposition between $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ and $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice. Therefore, although *ERα* deletion in B cells prolonged survival, these mice still developed fatal glomerulonephritis.

Higher levels of dsDNA IgG autoantibodies are associated with increased disease severity in lupus-prone mice. (NZB x NZW)F1 mice of both sexes with B cell *ERα* deletion have significantly lower levels of pathogenic anti-dsDNA IgG antibodies than mice with intact B cell *ERα*. Some isotypes of dsDNA antibodies are particularly pathogenic, especially the IgG2a and IgG2b isotypes which cause immune activation through multiple pathways (Azeredo da Silveira, 2002). $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice of both sexes have higher levels of anti-dsDNA IgG2a than $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice, and males have more anti-dsDNA IgG2b. Therefore, B cell specific loss of *ERα* causes a significant decrease in the levels of pathogenic dsDNA autoantibodies.

To determine if the decreased levels of autoantibodies in $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice were unique to dsDNA specific antibodies or were the result of general suppression of the immune response, we measured the amount of total serum IgM and IgG. The levels of total IgM and IgG are not different in $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ and $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice. This

indicates that the reduced levels of autoantibodies in $CD19^{Cre/+}$; $ER\alpha^{fl/-}$ mice are not due to general immunosuppression, but are the result of the loss of *ERα* signaling.

It has been shown that global *ERα* knockout does not affect the proportion of each B cell population in the bone marrow (Thurmond, 2000). Therefore, we examined the populations of developing B cells in the bone marrow to see if this was also the case in mice with *ERα* deletion in B cells. B cells first express *CD19* at the pro-B cell stage, so the populations we examined have all undergone at least some cre-mediated deletion of *ERα*. The only differences that we observed in B cell development in the bone marrow were due to the *CD19-Cre* knockin allele. In female mice, *CD19-Cre* caused an increase in the pre-B cell population and a decrease in immature B cells, but these populations were not affected in males. In *mb1-Cre* mice, which contain a different B cell specific driver of cre which is expressed in very early pro-B cells, there were no changes observed in bone marrow B cell populations on the BALB/c genetic background, although the sex of mice was not reported (Hobeika, 2006). These results suggest that *CD19* heterozygosity affects B cell development in females.

Additionally we sought to determine if B cell *ERα* impacted the follicular and marginal zone B cell populations in the spleen. In (NZB x NZW)F1 mice, the marginal zone B cell population was enriched for autoreactive B cells (Enghard, 2011). Studies with a different model have shown that an increased marginal zone population corresponds to an increase in the number of autoreactive B cells (Grimaldi, 2001). To the best of our knowledge, the effect of global *ERα* knockout on follicular and marginal zone B cell populations has not been previously reported. In female $ER\alpha^{-/-}$ (NZB x NZW)F1 mice, the marginal zone population was significantly increased. This was somewhat surprising because $ER\alpha^{-/-}$ (NZB x NZW)F1 females have significant attenuation of lupus (Bynote, 2008). There were no changes in these populations in $ER\alpha^{-/-}$ male mice.

The follicular and marginal zone B cell populations in the spleens of $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ and $CD19^{Cre/+}$; $ER\alpha^{fl/+}$ mice were examined to determine if *ERα* deletion in B cells affected these populations. *CD19-Cre* increased the proportion of follicular B cells and decreased the proportion of marginal zone B cells in both sexes of mice, but B cell specific deletion of *ERα* did not impact these populations. These results from global *ERα* knockout and B cell *ERα* deletion mice contradict the currently accepted dogma that an increase in the marginal zone B cell population corresponds with an increased population of activated autoreactive B cells and increased disease activity. One possible explanation is that the marginal zone B cells in *ERα* knockout females may be composed of cells which are resistant to activation or anergic. It has been shown that *ERα* knockout prevents B cell activation (Yoachim, 2015), but resistance to activation has not been tested in these mice. In *mb1-Cre* mice, no changes in the splenic B cell populations were found (Hobeika, 2006). This suggests that the alterations in these populations in *CD19-Cre* mice may be due to *CD19* heterozygosity. Unfortunately, the effect of *CD19* heterozygosity on splenocytes has not been reported in the literature.

The expression of *ERα* in B cells did not affect T cell activation in the spleen, but it did cause a significant decrease in activated splenic B cells in female mice. This effect was not observed in young male mice, but a difference in activation may develop at a later stage in males. Our data suggests that the significant decrease in activated B cells in female mice is the cause of attenuated autoantibody production, delayed development of glomerulonephritis, and attenuated mortality.

B cell specific deletion of *ERα* prolongs median survival by 68 days in females and 72 days in males. In mice with global *ERα* knockout, the median survival of females was increased by more than 219 days, and the median survival of males was increased by 85-113 days (Bynote, 2008). Compared to female mice, male mice with B cell *ERα*

deletion come closer to recapitulating the complete attenuation of lupus observed in *ERα* knockout mice. In female *ERα* knockout mice, serum levels of E2 are elevated by 10-fold, which likely causes increased ERβ signaling (Couse, 1995). Previous studies with (NZB x NZW)F1 mice have suggested that ERβ may slightly attenuate lupus (Li, 2007). In the high-E2 environment of *ERα* knockout female mice, this modest effect may be exaggerated, and be responsible for part of the attenuation of lupus seen in these mice. In any case, it is clear that deletion of *ERα* in B cells significantly ameliorates lupus in both female and male (NZB x NZW)F1 mice.

In conclusion, these studies showed that loss of ERα in a moderate proportion of B cells significantly attenuated lupus in (NZB x NZW)F1 mice. *ERα* deletion in B cells significantly decreased the production of autoantibodies and prolonged survival in both female and male mice. A decrease in B cell activation was found in young female mice with B cell specific *ERα* deletion. We propose that an even more dramatic attenuation of lupus would likely be observed if there was greater efficiency of *ERα* deletion in B cells. Although the low efficiency of B cell specific *ERα* deletion in this model was unintended, the significant reduction in lupus observed in these mice suggests that disruption of *ERα* signaling in B cells may be a reasonable target for the treatment of lupus.

General Discussion and Conclusion

Lupus is an autoimmune disease characterized by the production of autoreactive antibodies against nuclear antigens, and aberrant immune activation. Estrogens, through activation of ERα, exacerbate lupus, but it is not known which cell type or types mediate this effect. Additionally, estrogens affect hematopoietic cells in ways that autoimmunity, but it is not known if these effects are hematopoietic cell intrinsic.

In the studies presented here, we sought to discover the cell type or types in which ERα signaling promotes lupus. We hypothesized that ERα promotes lupus in B cells, and more generally, in hematopoietic cells. To test this hypothesis, we created two different mouse models on the (NZB x NZW)F1 lupus-prone genetic background that allowed us to assess the effect of *ERα* knockout in all hematopoietic cells, and the effect of B cell specific *ERα* deletion.

In chapter 3, we presented data from our studies that sought to determine the impact of *ERα* in hematopoietic cells on lupus. To address this, we created chimeric mice with $ERα^{-/-}$ or $ERα^{+/+}$ hematopoietic cells in $ERα^{+/+}$ lupus-prone (NZB x NZW)F1 mice. When characterizing this model, we observed that ERα in hematopoietic cells has a significant impact on the ability of hematopoietic cells to successfully reconstitute the bone marrow in female mice. A smaller proportion of $ERα^{-/-}$→$ERα^{fl/+}$ transplanted female mice had successful engraftment compared to $ERα^{+/+}$→$ERα^{fl/+}$ transplanted females. These grafts were stable, without a significant increase in the proportion of host $ERα^{fl/+}$ hematopoietic cells, until at least 6 months after transplant. Both $ERα^{-/-}$→$ERα^{fl/+}$ and $ERα^{+/+}$→$ERα^{fl/+}$ male chimeras had high rates of successful engraftment. These results indicate that $ERα^{-/-}$ hematopoietic cells from female mice are not able to reconstitute the hematopoietic compartment as efficiently as $ERα^{+/+}$ cells. Estrogen signaling through ERα plays an important role in the proliferative capacity of hematopoietic stem cells.

ERα$^{-/-}$ bone marrow cells do not repopulate the bone marrow as rapidly as *ERα*$^{+/+}$ cells after transplantation due to the reduced population size and proliferative capacity of *ERα*$^{-/-}$ HSCs in female mice (Sanchez-Aguilera, 2014; Nakada, 2014). Therefore, the lower proliferative capacity of HSCs from *ERα*$^{-/-}$ female mice could be the cause of the decreased engraftment efficiency that we observed.

In addition to proliferative capacity, to achieve successful engraftment, HSCs must be able to localize to the stem cell niche in the bone marrow. A defect in HSC homing could be responsible for the decreased engraftment efficiency in ERα$^{-/-}$→ERα$^{fl/+}$ female mice. To investigate this, we examined the expression of *CXCR4*, *CXCR7*, and *CXCL12* in bone marrow from *ERα*$^{+/+}$ and *ERα*$^{-/-}$ mice. CXCR4 is a G-protein coupled chemokine receptor expressed on the surface of hematopoietic stem and progenitor cells. HSCs are guided to the niche stem cell niche by the CXCR4 ligand, CXCL12 (also called stromal-derived factor 1), which is secreted by bone marrow stromal cells. The concentration of CXCL12 is higher in the bone marrow than in the peripheral blood, and CXCR4-expressing HSCs locate the stem cell niche based on this gradient. CXCL12 can also bind CXCR7, a scavenger receptor which targets CXCL12 for degradation, and thus prevents CXCL12 from binding CXCR4 (Naumann, 2010). The balance between CXCR4 and CXCR7 expression regulates HSCs' ability to hone to the bone marrow stroma. Estrogen increases the expression of *CXCL12* and *CXCR4*, and decreases the expression of *CXCR7* (Boudot, 2011; Li, 2013). The CXCR4-CXCL12 interaction is not the only factor involved in HSC homing, but it plays a significant role in the engraftment potential of transplanted hematopoietic cells (Adamiak, 2015). Our analysis of the mRNA levels of these chemokine receptors and ligand did not find a difference in the expression of these molecules between *ERα*$^{+/+}$ and *ERα*$^{-/-}$ mice of either sex. These

results do not preclude the possibility that other factors that affect HSC homing could be affected by the loss of *ERα* in females.

Despite a lower than expected rate of success in producing $ERα^{-/-}→ERα^{fl/+}$ female mice with high levels of engraftment, we were able to produce enough successfully transplanted mice to study the effects of *ERα* in hematopoietic cells on lupus. The survival of $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ chimeric mice was monitored for up to one year. There was no difference in the median lifespan of $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ female mice. Additionally, there was no difference in median survival between $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ males. This suggests that *ERα* in hematopoietic cells does not affect the rate of lupus-related mortality in either female or male mice.

In addition to its effects on survival, we examined the effects of *ERα* in hematopoietic cells on the production of autoantibodies. Autoantibody production was measured in $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ chimeric mice. We found that female $ERα^{-/-}→ERα^{fl/+}$ mice produced less anti-chromatin IgG and anti-dsDNA IgG at 6 months of age. By 7 months of age, there was no difference in the amount of these autoantibodies produced by $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ mice. This suggests that mice with $ERα^{-/-}$ hematopoietic cells have attenuated autoantibody production at a young age, but that loss of *ERα* does not protect against autoantibody production as the mice age. The rate of engraftment in these mice remained high for at least 6 months after the transplant (about 8 months old), which suggests that progressive graft failure is not the cause of increased autoantibody production in $ERα^{-/-}→ERα^{fl/+}$ female mice. We hypothesized that there may be some pathogenic element of the host immune system that remained in $ERα^{-/-}→ERα^{fl/+}$ mice which was able to overcome the protective effects of $ERα^{-/-}$ hematopoietic cells and cause increased immune activation in these mice.

Short-lived plasmablasts and long-lived plasma cells in (NZB x NZW)F1 mice are resistant to depletion by cyclophosphamide (Hoyer, 2004), which suggests that these cells may be able to survive irradiation. When we examined the DNA of plasma cells isolated from mice at the end of the survival study, many months after irradiation, we were able to detect host plasma cell DNA in many mice. Additionally, we isolated dsDNA-reactive long-lived and short-lived plasma cells from 10 week old mice that had been irradiated and not given replacement bone marrow. 0.27-0.41% of the plasma cells isolated from these mice were dsDNA-reactive, which is a large percent of cells of a single reactivity. These results show that a significant population of pathogenic dsDNA reactive plasma cells are present in pre-autoimmune (NZB x NZW)F1 mice and that these cells survive irradiation. Therefore, chimeric $ERα^{-/-}→ERα^{fl/+}$ and $ERα^{+/+}→ERα^{fl/+}$ mice are likely to have a significant population of autoreactive plasma cells from the host. These host-derived autoreactive plasma cells may be sufficient to drive the progression of lupus in $ERα^{-/-}→ERα^{fl/+}$ mice. The $ERα^{+/+}→ERα^{fl/+}$ chimeric mice had $ERα^{+/+}$ plasma cells from both the host and the donor hematopoietic cells, and produced more autoantibodies initially. The delayed antibody production in $ERα^{-/-}→ERα^{fl/+}$ female mice could be from activation of autoreactive host-derived $ERα^{fl/+}$ plasma cells, and not $ERα^{-/-}$ cells. This would suggest that $ERα^{-/-}$ hematopoietic cells do attenuate lupus in (NZB x NZW)F1 mice, but in this chimeric model, these effects are masked by the activity of residual dsDNA-reactive $ERα^{fl/+}$ plasma cells.

Although we had hypothesized that *ERα* in hematopoietic cells impacts lupus, *ERα* is widely expressed by cells throughout the body, and the expression of *ERα* in other cell types could impact lupus. For instance, *ERα* could impact lupus through bone marrow stromal cells (BMSCs). BMSCs support hematopoietic cells both through direct contact

and through the secretion of growth factors. BMSCs express *ERα*, and estrogen regulates the ability of BMSCs to support hematopoietic cells (Smithson, 1995).

In addition to the chimeras created to study the role of *ERα* in hematopoietic cells on lupus, we also created chimeric mice designed to address the role of *ERα* in non-hematopoietic cells on lupus. To do this, we transplanted (NZB x NZW)F1 $ERα^{+/+}$ hematopoietic cells into $ERα^{-/-}$ or $ERα^{+/-}$ (NZB x NZW)F1 recipients. Studies with these mice revealed that loss of *ERα* in non-hematopoietic cells has a significant, negative effect on engraftment in female mice. The transplant efficiency in female $ERα^{+/+}→ERα^{-/-}$ mice was dramatically lower than in female $ERα^{+/+}→ERα^{+/-}$ mice. The proportion of $ERα^{-/-}$ hematopoietic cells in $ERα^{+/+}→ERα^{-/-}$ females increased significantly over time, indicating progressive failure of the donor cells. Male $ERα^{+/+}→ERα^{-/-}$ and $ERα^{+/+}→ERα^{+/-}$ chimeras had successful transplants that were stable over time. Again, we observed that ERα impacted the success of hematopoietic engraftment in female mice and not males. In this case, the hormonal environment in $ERα^{-/-}$ females likely leads to exhaustion of the transplanted HSCs, and failure of $ERα^{+/+}$ HSC grafts in $ERα^{-/-}$ recipient females.

Elevated levels of estrogen significantly affect the proliferation of HSCs. A moderate increase in serum E2, like the approximately 2-fold increase that occurs during pregnancy, causes HSCs to divide more frequently, and causes an increase in the number of HSCs (Nakada, 2014). However, in conditions of high serum E2, the HSC population decreases, likely as the result of exhaustion due to rapid division (Thurmond, 2000). After chronic exposure to estrogen, HSCs are unable to effectively repopulate some hematopoietic populations (Illing, 2012). Female *ERα* knockout mice have serum E2 levels about 10x higher than $ERα^{+/+}$ females (Eddy, 1996). Therefore, in female $ERα^{+/+}→ERα^{-/-}$ mice, the donor $ERα^{+/+}$ HSCs are likely exhausted after rapid estrogen-induced proliferation, which causes the proportion of $ERα^{+/+}$ cells in the hematopoietic

population to progressively decrease. Male *ERα* knockout mice do not have elevated levels of E2, so $ERα^{+/+}$ hematopoietic cells would not undergo rapid proliferation in $ERα^{-/-}$ males.

Examining the rate of hematopoietic engraftment in chimeric mice revealed that the *ERα* genotype of both donor and host play a significant role in the success of hematopoietic reconstitution in females, but not males. Female $ERα^{-/-}$→$ERα^{fl/+}$ chimeras have a lower rate of successful reconstitution than $ERα^{+/+}$→$ERα^{fl/+}$ females. Female $ERα^{+/+}$→$ERα^{-/-}$ chimeras have a dramatically lower rate of engraftment than $ERα^{+/+}$→$ERα^{+/-}$ chimeras which progressively decreases over time due to exhaustion of the HSC population.

Transplantation of hematopoietic cells has been investigated as a treatment for many diseases, particularly cancers. The use of high dose chemotherapy followed by autologous stem cell transplant (HDCT/ASCT) has been investigated as a treatment for breast cancer with mixed results. The amount of estrogen in breast cancer patients could impact the engraftment of the transplanted hematopoietic cells, and the ability of immune cells to fight cancer cells. Breast cancer patients can be either pre- or post-menopausal and some receive fulvestrant treatment, which makes it difficult to determine the estrogen level in patients and how this affects the success of the HDCT/ASCT treatment. Phase I/II trials of HDCT/ASCT were done in young, premenopausal women and showed promise for treating breast cancer, but later trials that included both premenopausal and postmenopausal women had mixed results. One study showed that postmenopausal women given HDCT/ASCR were more likely to die of tumor-related death than premenopausal women (Wild, 2004). Although far from conclusive, these results may indicate that low levels of estrogen negatively affect the effectiveness of this transplantation therapy. If this is true, it is likely related to the effects of estrogen on hematopoietic cell division.

In our experiments, because of the unsuccessful engraftment of ERα$^{+/+}$→ERα$^{-/-}$ female mice, we were unable to use these female mice to draw conclusions about the role of ERα in non-hematopoietic cells in lupus. The median survival times of male ERα$^{+/+}$→ERα$^{-/-}$ and ERα$^{+/+}$→ERα$^{+/-}$ mice are not different. Therefore, the *ERα* genotype of non-hematopoietic cells does not have an impact on survival in males.

The development of fatal glomerulonephritis is characteristic of lupus in (NZB x NZW)F1 mice. Therefore, when chimeric mice died or were sacrificed at the end of the one year survival study, kidneys were collected and evaluated for nephritis. All chimeric mice from each group had moderate to severe glomerulonephritis. This is similar to what was observed in *ERα*$^{+/+}$ (NZB x NZW)F1 mice, where all female and male mice developed severe glomerulonephritis by the end of an 18 month survival study (Bynote, 2008).

Although glomerulonephritis and autoantibody production are characteristic of lupus in (NZB x NZW)F1 mice, they can also be symptoms of graft versus host disease (GVHD), a condition that can develop after transplantation of hematopoietic cells. GVHD occurs when there is a mismatch in histocompatibility loci between the donor and recipient, which causes the donor hematopoietic cells to launch an immune response against host cells. Acute GVHD (aGVHD) manifests immediately after transplantation and causes severe skin rash and diarrhea. Murine models of aGVHD result in continual weight loss and death by one month after transplant (Lin, 2014). Chronic graft vs host disease (cGVHD) develops later, and can cause autoantibody production and glomerulonephritis (Fraile, 2013). In our chimeric mouse model, both the donor and recipient mice are (NZB x NZW)F1s and should not have any mismatches at histocompatibility loci. Although the chimeric mice produced for these studies initially lost weight after the transplant, they gained back almost all of this weight within 2 weeks. Additionally, no diarrhea or skin manifestations were observed in these mice over the course of the study. In these

chimeric (NZB x NZW)F1 mice, significant sex-specific survival differences were observed, which is characteristic of lupus, and not GVHD. Therefore, we are confident that these mice did not develop GVHD, and the autoantibody production and glomerulonephritis that occurred in these mice were due to lupus.

In conclusion, although we were not able to show that *ERα* in hematopoietic cells significantly affected lupus, the persistence of host-derived long-lived plasma cells, combined with the partial attenuation of autoantibody production in $ERα^{-/-}→ERα^{fl/+}$ females suggests that a small population of $ERα^{+/+}$ dsDNA-reactive plasma cells may be sufficient to cause lupus in (NZB x NZW)F1 mice. These studies also demonstrated that estrogen signaling, through ERα, in hematopoietic and non-hematopoietic cells is important for successful engraftment after transplantation in females.

In addition to investigating the impact of *ERα* in hematopoietic cells on lupus, we were also interested in examining the effects of *ERα* in B cells on lupus. High levels of estrogen promote the development of high-affinity autoreactive B cells through *ERα*. Activated autoreactive B cells become plasma cells that produce pathogenic autoantibodies. These pathogenic autoantibodies form immune complexes, which cause inflammation and tissue damage in lupus patients.

In chapter 4, we presented data from our studies on lupus-prone (NZB x NZW)F1 mice with B cell specific deletion of *ERα*. To achieve B cell specific deletion of *ERα*, a cre-loxP system was used in which the *CD19-Cre* knockin allele, which is expressed in B cells, caused the deletion of a floxed allele of *ERα*. The efficiency of *ERα* deletion by *CD19-Cre* was much lower than has been previously reported, with only ~50% deletion of *ERα* in $CD19^{+}$ splenocytes. Another study from our lab which used *Lck-Cre* to cause *ERα*

deletion in T cells also found that the deletion efficiency was lower on the (NZB x NZW)F1 genetic background than had been previously reported (Nelson, 2016).

Mammalian cells contain cryptic or pseudo loxP sites, which can deviate significantly from the consensus loxP sequence and be cleaved by cre recombinase (Thyagarajan, 2000). Defects in double strand break repair are associated with lupus, and unrepaired dsDNA breaks can lead to apoptosis. (NZB x NZW)F1 mice with the *CD19-Cre* knockin allele have a small but significant increase in apoptotic $CD19^+$ splenocytes compared to mice without cre. (NZB x NZW)F1 mice with *Lck-Cre* also have a significant increase in apoptotic T cells (Nelson, 2016). Cells that have high enough cre expression to cause deletion of *ERα* may also have DNA damage at pseudo loxP sites caused by cre. Unrepaired DNA damage leads to apoptosis, and could contribute to the moderate deletion efficiency we observed in (NZB x NZW)F1 mice.

Another possible reason for the moderate deletion efficiency of *ERα* observed in these mice would be if B cells that lacked *ERα* expression were at a survival disadvantage. We found that the rate of deletion of the floxed *ERα* allele was the same in B cells that had total loss of *ERα* in B cells, and those that retained one functional copy of *ERα* after recombination. Therefore, *ERα* knockout B cells were not at a survival disadvantage, and the reduced proportion of $CD19^+$ cells that underwent cre-mediated deletion was likely an effect of the (NZB x NZW)F1 genetic background.

Despite the moderate rate of *ERα* deletion, our studies showed that loss of *ERα* in a moderate proportion of B cells caused a significant improvement in lupus. Young female mice with B cell *ERα* deletion had a lower percentage of activated B cells compared to *CD19-Cre* controls. Therefore, deletion of *ERα* in B cells prevented B cell activation in pre-autoimmune mice. Although we did not observe a difference in B cell activation in

young male mice, we predict that there would be a difference in slightly older male mice, since males have a longer latency to disease than females.

In addition to decreased activation of B cells, mice of both sexes with B cell *ERα* deletion had significantly lower levels of pathogenic anti-dsDNA IgG autoantibodies than *CD19-Cre* only controls. There was no difference in the production of total IgM or IgG, which indicates that the reduction in anti-dsDNA IgG is particular to this antibody specificity and is not due to general immunosuppression from loss of *ERα* in B cells.

Lower levels of pathogenic autoantibodies in mice with B cell *ERα* deletion caused a delay in the development of fatal glomerulonephritis. Both female and male mice with B cell *ERα* deletion had longer median survival than *CD19-Cre* only controls. The differences in median survival between female and male (NZB x NZW)F1 mice with B cell specific *ERα* deletion and *CD19-Cre* controls are 68 days and 72 days, respectively. Therefore, partial deletion of *ERα* in B cells attenuated lupus-related mortality by a similar amount of time in females and males.

Female (NZB x NZW)F1 mice with whole-body *ERα* knockout had a far longer attenuation of lupus than the 68 days in females with B cell specific deletion of *ERα* (Bynote, 2008). However, this study was complicated by the hormonal changes associated with *ERα* knockout in females. Female *ERα* knockout mice have about 10x more serum E2 than $ERα^{+/+}$ mice, while there is no change in serum E2 in *ERα* knockout males (Eddy, 1996). The increased level of serum E2 in (NZB x NZW)F1 females would have caused increased *ERβ* activation. It has been suggested that ERβ activation has a moderate protective effect in (NZB x NZW)F1 mice (Li, 2007), and this could contribute significantly to the attenuation of lupus in (NZB x NZW)F1 *ERα* knockout females. The attenuation of lupus in male *ERα* knockout mice is 85-113 days, which is similar to the

attenuation of 72 days in males with B cell specific *ERα* deletion (Bynote, 2008). So, even though we did not observe a sex-specific difference in the attenuation of lupus in mice with partial *ERα* deletion in B cells, this is not necessarily inconsistent with past studies, because female mice with partial *ERα* deletion in B cells should not have hormonal abnormalities like global *ERα* knockout females.

There are several studies that we would like to perform to further characterize this model. To determine if the development of glomerulonephritis is indeed attenuated in mice with B cell specific ERα deletion, we will collect kidneys from female and male mice at a pre-defined age, after initiation of disease, but before mice begin to succumb to disease. These kidneys will be evaluated for glomerulonephritis and immune complex deposition. At this time, we will also analyze B cell activation in male mice to see if males with B cell *ERα* deletion have a lower proportion of activated B cells. It has been reported that estrogen, through activation of *ERα*, causes an increase in the frequency of Ig-producing B cells (Erlandsson, 2003). To see if *ERα* deletion in B cells causes a change in the total number of autoantibody producing B cells, an ELISpot assay will be performed.

The studies presented here suggest that *ERα* in B cells may be an attractive target for lupus therapy. Although unintended, the modest efficiency of *ERα* deletion in this model suggests that disruption of ERα signaling in only a portion of B cells is sufficient to cause significant attenuation of lupus. Therefore, after the studies to further characterize this model have been completed, the next step is to begin designing a targeted method to deliver an ERα blocking molecule to B cells. It has been shown that liposomes loaded with doxorubicin can be targeted to B cells with anti-CD19 antibodies. A similar design could be used to encapsulate an anti-estrogen, such as fulvestrant, for delivery to B cells.

In addition to the effects of B cell specific deletion of *ERα* on lupus, we also observed many changes in our experimental mice that can be attributed to the *CD19-Cre* knockin allele. In both female and male mice, the survival of *CD19-Cre* only control mice was significantly shorter than $CD19^{+/+}$ mice. We were not able to determine if this was an effect of *CD19* heterozygosity, cre recombinase expression, or a combination of both. CD19 promotes BCR signaling (Depoil, 2008), and *CD19-Cre* mice have about half the *CD19* expression as $CD19^{+/+}$ mice, which could lead to less effective BCR signaling, and increased survival of autoreactive B cells. Alternatively, a cre-induced increase in apoptosis could cause the release of nuclear antigens and increased activation of autoreactive B cells. In support of this, an increase in apoptosis was observed in $CD19^{+}$ splenocytes from mice with the *CD19-Cre* knockin allele. Whatever the cause, the *CD19-Cre* knockin allele had significant effects on mortality in these studies. This emphasizes the need for appropriate cre-only controls in experiments using the cre-loxP system. Without comparing the survival of these two groups, we would have incorrectly concluded that B cell specific deletion of *ERα* did not affect the survival of lupus-prone mice.

The results of the two studies presented here appear contradictory. The study presented in chapter 3 failed to show that *ERα* in hematopoietic cells promotes lupus, while the study presented in chapter 4 showed that deletion of *ERα* in a moderate proportion of B cells significantly attenuates lupus. There are a few possible explanations for this disconnect.

The hematopoietic compartment is made up of many types of cells, which play different roles in immunity. *ERα* is widely expressed in hematopoietic cells from HSCs to fully differentiated cells, and activation of ERα causes varied effects in different cell types. Consequently, the loss of *ERα* in various immune cell types will have different effects.

Our studies have shown loss of *ERα* in even a portion of B cells leads to a lower level of immune activation. However, the loss of *ERα* in a different cell type, like Tregs, which negatively regulate the immune response, could lead to unchecked immune activation. E2 causes an increase in the Treg population, and also increases the suppressor capacity of Tregs via multiple mechanisms (Polanczyk, 2004; Prieto, 2006; Polanczyk, 2007). Some of these effects are mediated by ERα. Therefore, it is possible that loss of *ERα* from the entire hematopoietic compartment affects some cells in a lupus-promoting and others in others in a lupus-attenuating manner, and that in the environment of an $ERα^{+/+}$ mouse, changes in the number or function of different types of immune cells results in no net change in the development of lupus. Thus, proper ERα signaling in Tregs may provide crucial protection from unattenuated lupus.

Another explanation for these results is that while *ERα* in hematopoietic cells promotes lupus, the chimeric (NZB x NZW)F1 mice produced for our studies did not have a pure enough population of hematopoietic cells to show an effect on survival. We have shown that $ERα^{+/+}$ host-derived dsDNA-reactive plasma cells survive irradiation in (NZB x NZW)F1 mice and can be detected months after the transplant. A large percentage of these surviving plasma cells were dsDNA-reactive. In an environment where most other hematopoietic cells are $ERα^{-/-}$, $ERα^{+/+}$ plasma cells could be free from the inhibitory signals that normally prevent high levels of activation. Activated dsDNA reactive plasma cells could then produce large amounts of autoantibodies, thus promoting lupus.

Conclusion

We created chimeric (NZB x NZW)F1 mice with different *ERα* genotypes in hematopoietic and non-hematopoietic cells to study the impact of ERα in these cells on lupus. Although we were not able to show that *ERα* in hematopoietic cells promotes lupus, our studies with these chimeric mice did show that ERα plays an important role in the successful engraftment of hematopoietic cells in female mice. Loss of *ERα* in hematopoietic cells led to decreased engraftment efficiency.

Additionally, to study the role of ERα in B cells in lupus, we created a (NZB x NZW)F1 model with *ERα* deletion specifically in B cells. Loss of *ERα* in a moderate proportion of B cells significantly reduced B cell activation and attenuated autoantibody production and mortality in (NZB x NZW)F1 mice. As a result of these studies, we have identified ERα in B cells as a potential new target for lupus therapy. Additionally, we have shown that the *CD19-Cre* knockin allele alone has significant effects in lupus-prone mice, emphasizing that cre-only controls are essential in experiments that utilize cre-loxP systems.

Although the results of these studies appear contradictory, there are a few possible explanations for these results. One possibility is that the hematopoietic compartment of the chimeric mice produced to study the role of *ERα* in hematopoietic cells was not composed of a pure population of cells from the donor mouse, and that residual host-derived cells were sufficient to cause lupus in chimeric mice. Another possibility is that by knocking out *ERα* from the entire hematopoietic compartment, both cells that promote and attenuate lupus were affected, leading to no net change in the development of lupus.

Appendix A: Dexamethasone Prodrug Treatment Prevents Nephritis in Lupus-prone (NZB×NZW)F1 Mice without Causing Systemic Side Effects

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Abstract

Objective

To evaluate the potentially improved therapeutic efficacy and safety of nephrotropic macromolecular prodrugs of glucocorticoids (GC) in the treatment of lupus nephritis.

Methods

Monthly injection of *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-based dexamethasone prodrug (P-Dex) and daily injection of dexamethasone phosphate sodium (Dex, overall dose equivalent to P-Dex) were given to lupus-prone (NZB×NZW)F1 female mice for two months. The animals were monitored for albuminuria, mean arterial pressure and serum autoantibody levels during the treatment. Nephritis, renal immune complexes and macrophage infiltration were evaluated histologically. The bone quality was analyzed with pDEXA and μ-CT. Optical imaging, immunohistochemistry (IHC) and fluorescence-activated cell sorting (FACS) were used to understand the *in vivo* distribution of P-Dex. The anti-inflammatory effect of P-Dex was validated using LPS-activated human proximal tubule epithelial cells (HK-2).

Results

Monthly P-Dex injection completely abolished albuminuria in the (NZB×NZW)F1 mice, which is significantly ($P < 0.001$) more efficacious than daily Dex treatment. P-Dex did not reduce serum levels of anti-dsDNA antibodies or renal immune complexes, but did reduce macrophage infiltration, a marker of chronic inflammation. IHC and FACS analyses revealed that P-Dex was primarily sequestered by proximal tubule epithelial cells and it could attenuate the inflammatory response in HK-2 cell culture. Different from

Dex treatment, P-Dex did not lead to any significant bone quality deterioration or total serum IgG reduction.

Conclusion

Macromolecularization of GCs renders them nephrotropic. The protracted retention, subcellular processing and activation of GC prodrugs by kidney cells would potentiate nephritis resolution with reduced risk of systemic toxicities.

Introduction

Lupus is an autoimmune disease in which autoantibodies are produced against nuclear antigens, including double stranded DNA (dsDNA). Renal deposition of anti-dsDNA IgG containing immune complexes leads to nephritis, a major cause of morbidity and mortality in lupus patients. Renal immune complexes induce inflammation and immune cell infiltration, which if unresolved, lead to renal injury, dysfunction, and failure. Nephritis is treated with glucocorticoids (GCs), which are suboptimal because they frequently cause off-target toxicity. Because lupus patients often take GCs continuously for many years, they are at high risk for developing GC-associated adverse side effects, including osteoporosis and immunosuppression.

The therapeutic efficacy of a drug depends on its specificity for its molecular target and its concentration at the site of interaction with the target. Advances in understanding lupus have stimulated progress in the identification of drugs that interact with molecular targets and pathways associated with disease [1]. These efforts, nevertheless, have not addressed the problems created by our inability to control the *in vivo* drug concentration at either the intended site(s) of action or off target sites, where drug action results in adverse side effects.

To address this challenge, we have developed a macromolecular prodrug of dexamethasone (P-Dex), which passively targets inflamed tissues and provides superior and sustained resolution of inflammation in several animal models [2–4]. Here, we demonstrate that P-Dex prevents nephritis in lupus-prone (NZB×NZW)F1 mice. P-Dex demonstrated reduced systemic toxicity compared to the equivalent dose of dexamethasone. Mechanistic studies indicate that the nephrotropism, cell-mediated local sequestration, subcellular processing and activation of P-Dex likely contribute to its superior therapeutic efficacy and reduced systemic toxicities.

Materials and Methods

Synthesis of macromolecular prodrugs

P-Dex (Figure 1A) was synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization as described previously [3]. Briefly, *N*-(2-hydroxypropyl)methacrylamide (HPMA), *N*-methacryloylglycylglycylhydrazyl dexamethasone (MA-Dex) [3] and other comonomers {*N*-methacryloylaminopropyl fluorescein thiourea [5] and *N*-(3-aminopropyl)methacrylamide hydrochloride (APMA, Polysciences, Inc. Warrington, PA)} were copolymerized at 40°C under Argon for 48 hours (h) with 2,2′-azobisisobutyronitrile as the initiator and *S*,*S*′-bis(α, α ′-dimethyl-α″-acetic acid) trithiocarbonate as the RAFT agent [6]. The resulting polymers were purified by LH-20 column (GE HealthCare, Waukesha, WI) and lyophilized. IRDye 800CW and Alexa Fluor® 488 labeled P-Dex (P-Dex-IRDye and P-Dex-Alexa) were obtained via polymer analogous reactions between poly(HPMA-*co*-MA-Dex-*co*-APMA) and NHS esters of these dyes [2].

Experimental animals and drug treatment

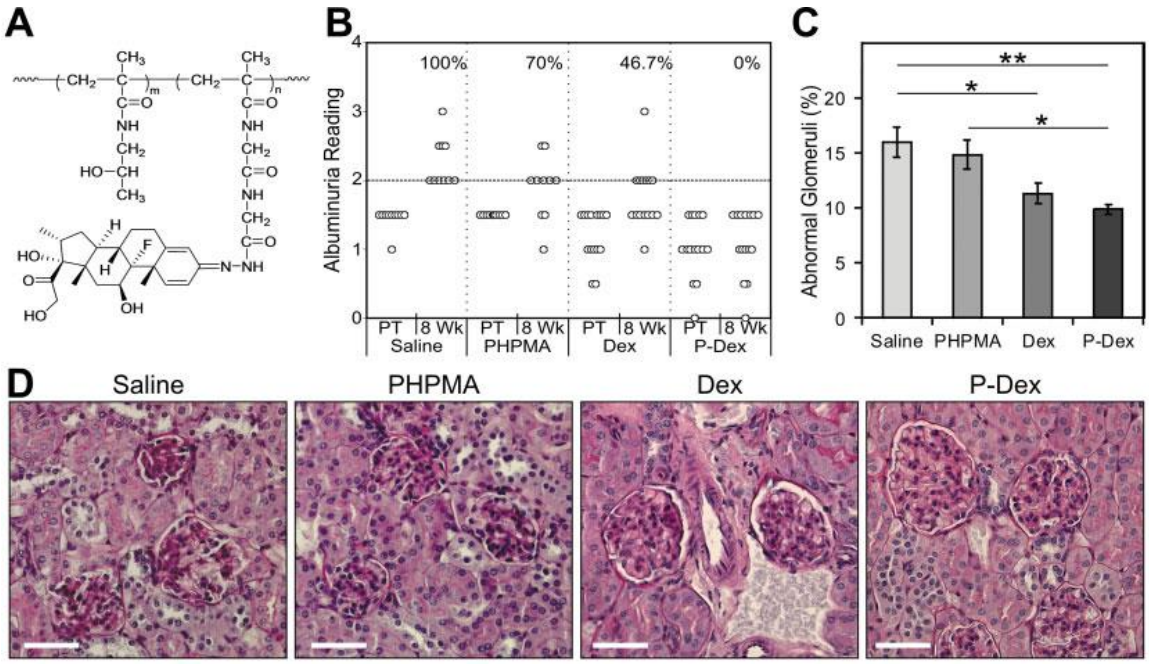

Figure 1. **Dexamethasone prodrug (P-Dex) prevents albuminuria and reduces glomerular damage in female (NZB x NZW)F1 mice.** A, Chemical structure of P-Dex. B, Albuminuria readings for mice in the saline (n =10), *N*-(2-hydroxypropyl)methacrylamide homopolymer (PHPMA) (n = 10), dexamethasone 21-phosphate disodium (Dex) (n = 15), and P-Dex (n = 15) treatment groups at the pretreatment (PT) and 8-week time points. The percentages shown are the incidence of albuminuria at the 8-week time point. Each data point represents an individual mouse. C, Quantification of the incidence of abnormal glomeruli in each treatment group. Values are the mean ± SEM. * = $P < 1 \times 10^{-3}$; ** = $P < 5 \times 10^{-4}$. D, Representative periodic acid–Schiff–stained histologic sections from each treatment group. Bars = 50 μm.

(NZB×NZW)F1 and NZW females (Jackson Laboratories, Bar Harbor, ME) were housed under controlled humidity, temperature and lighting conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 1996). Mice were given Harlan irradiated rodent diet 7904 (Harlan Teklad, Madison, WI) and allowed to feed *ad libitum*. All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

At 16 weeks of age, mice were treated via i.v. injection with saline, *N*-(2-hydroxypropyl)methacrylamide homopolymer (PHPMA), or P–Dex (250 mg/kg) every 4 weeks. A fourth group of mice were given daily i.p. injections of dexamethasone 21-phosphate disodium (Dex, 1.32 mg/kg, containing 1.00 mg/kg of dexamthasone, Hawkins, Inc., Minneapolis, MN). Dosages of Dex and P-Dex were calculated and prepared as such that mice received the same dose of dexamethasone over the 8-week treatment period.

Mice were monitored weekly for albuminuria using Albustix (Siemens Corp., Washington DC). Albuminuria was defined as two consecutive 2+ readings (100 mg/dL). Every 4 weeks, serum was isolated from peripheral blood, and mean arterial pressure (MAP) was recorded via tail-cuff method using the CODA blood pressure measuring system and software (Kent Scientific, Torrington, CT). One week after cessation of treatment, mice were euthanized and tissues harvested.

Analysis of nephritis, renal immune complexes and macrophage infiltration

Kidneys were fixed, paraffin-embedded, sectioned and stained with Periodic Acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MO) and analyzed by light microscopy. One hundred glomeruli per mouse were evaluated as described previously [7].

Renal immune complexes were visualized by immunohistochemistry. After deparaffinization and rehydration, slides were incubated in H_2O_2, washed and incubated in citrate buffer (Vector Laboratories, Burlingame, CA). Slides were blocked with normal horse serum (Vector Labs), and incubated with anti-mouse IgG (Vector Labs). Antibody binding was visualized using Vectastain Elite reagents (Vector Labs). Staining intensity (represented as arbitrary gray units or AGU) of fifty glomeruli per mouse was quantified using Axiovision software (v4.6.3.0; Carl Zeiss, Thornwood, NY). A second set of slides stained for immune complexes was counterstained with hematoxylin; these slides were for illustration purposes only.

Renal macrophage infiltration was assessed via immunofluorescence with the macrophage marker Iba-1 (Biocare Medical, Concord, CA) as described previously [8], with an added blocking step in Sudan Black B. Staining was visualized and quantitated using confocal microscopy and Zen 2010 software (v6; Carl Zeiss).

Serological analysis of serum immunoglobulin levels

Serum immunoglobulin concentrations were determined by ELISA (Southern Biotech, Birmingham, AL). The IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 levels were added together to obtain total serum IgG levels. Serum Anti-dsDNA IgG levels were determined by ELISA (Alpha Diagnostics International, San Antonio, TX) as described previously [7].

Analysis of bone quality

Femoral bone mineral density (BMD) and micro-architectural parameters were measured as described previously [9], using pDEXA® Sabre™ X-ray bone densitometer (Norland Medical System, Inc, Fort Atkinson, WI) and Skyscan 1172 micro-CT system (Skyscan, Kontich, Belgium), respectively. pDEXA analysis was performed with a 20 mm/sec scanning speed and a 0.2 × 0.2 mm resolution. Areal BMD was calculated using SABRE RESEARCH software (v3.9.4). Micro-CT scanning parameters were: voltage, 55 kV; current, 189 μA; exposure time, 230 ms; resolution, 6.2 μm; and aluminum filter (0.5 mm). Three-dimensional reconstructions were performed with NRecon and Dataviewer software (Skyscan). Trabecular bone was selected for analysis by a polygonal region of interest within the center of the femur, starting at 20 slices (0.25 mm) proximal from the growth plate and extending proximally 80 slices (0.99 mm) further. Trabecular bone volume fraction, number and thickness were quantified with CTAn software (Skyscan).

Near infrared imaging analysis

Mice received P-Dex-IRDye (148 nmol IRDye per kg body wt.) by i.v. injection. Mice were euthanized 2 or 7 days (d) later and tissues were harvested and imaged using an XENOGEN IVIS® 200 Series Imaging System (Caliper Life Sciences, Hopkinton, MA).

Immunohistochemical analyses of P-Dex distribution within kidney

Mice were given P-Dex-Alexa (300 nmol Alexa Fluor® 488 per kg body wt.) via i.v. injection. Seven days later, mice were perfused and euthanized. Kidneys were fixed, paraffin-embedded and sectioned. For direct staining, APC-labeled anti-mouse B220 and CD8a, and PE-labeled anti-mouse CD31 and CD4 (BD Pharmingen, San Diego, CA) antibodies were used. For indirect staining, sections were incubated with anti-mouse CD11c (eBioscience, San Diego, CA), E-cadherin (R&D Systems, Minneapolis,

MN), F4/80 and Ly-6G (eBioscience) antibodies, followed with PE-labeled secondary antibody (eBioscience; R&D Systems; and Invitrogen, Carlsbad, CA). Stained sections were examined under a Nikon Swept Field confocal microscope (Nikon Instruments Inc, Melville, NY).

Flow cytometry

Mice received an i.v. injection of P-Dex-Alexa. At necropsy (7 d post injection), white blood cells (WBCs) were isolated from peripheral blood. Mice were perfused, and tissues were isolated, macerated and passed through a 70-μm strainer to obtain single cell suspensions. Cells were analyzed with Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The following antibodies were used: APC-labeled anti-mouse B220, CD19, CD5 and CD8a; PE-labeled anti-mouse F4/80 (eBioscience), Ly6B.2 (AbD Serotec, Raleigh, NC), CD4, CD138, CD31 and IgD (BD Pharmingen); PECy7-labeled anti-mouse IgM (BD Pharmingen); anti-mouse CD11c, α-smooth muscle actin and E-cadherin followed with PE-labeled secondary antibody.

Cell culture

Human proximal tubule epithelial cells (HK-2) were grown in RPMI 1640 with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (0.1 mg/mL). To investigate the internalization of fluorescein isothiocyanate (FITC) labeled P-Dex (P-Dex-FITC), HK-2 cells were stimulated overnight with LPS (10 μg/mL). P-Dex-FITC (final concentration 200 μg/mL) was added to LPS-stimulated and untreated HK-2 cells. After specified intervals, cells were rinsed and analyzed by FACS.

For subcellular localization studies, HK-2 cells were cultured overnight with LPS and then incubated with P-Dex-FITC (200 μg/mL) for 24 h. Cells were rinsed and incubated

with 75 nM Lysotracker DND-99 (Invitrogen) for 3 h. After rinsing, cells were stained with DAPI, fixed, mounted and observed by confocal microscopy.

To analyze the anti-inflammatory effect of P-Dex, HK-2 cells were treated with LPS plus Dex (2 μM) or P-Dex (2 μM dexamethasone equivalent) for 24 h. Supernatants were collected and stored at −80°C. Cells were rinsed and incubated with fresh medium containing LPS for an additional 48 h. Supernatants were collected and assayed for IL-6 level by ELISA (R&D Systems).

Statistical methods

Comparisons were performed using Fishers exact test, Wilcoxon signed-ranks test, Mann-Whitney U test, independent or paired samples t-test, or one-way ANOVA with Tukey's *post hoc* test where appropriate. Statistical analyses were performed using SPSS software (version 19.0). A two-sided $P \leq 0.05$ was considered significant. Two-sided p-values are provided. Mean ± standard error of the mean is presented.

Results

P-Dex prevents albuminuria and reduces glomerular damage

Albuminuria was measured in (NZB×NZW)F1 mice to assess nephritis-associated loss of renal function. Prior to treatment, none of the mice displayed albuminuria. However, after 8 weeks, 100% of saline treated mice and 70% of PHPMA treated mice exhibited albuminuria (Figure 1B). The incidence of albuminuria in these groups did not differ significantly ($P = 0.2$). After eight weeks, 47% of Dex treated mice displayed albuminuria (Figure 1B), which was significantly different from the saline ($P < 0.01$), but not the PHPMA group ($P = 0.4$). Strikingly, after eight weeks, 0% of P-Dex treated mice

exhibited albuminuria (Figure 1B), which is significantly different from the saline ($P < 5\times10^{-7}$), PHPMA ($P < 5\times10^{-4}$) and Dex ($P < 5\times10^{-2}$) groups. Thus, P-Dex was more effective than Dex in preventing albuminuria.

To further assess renal function, PAS-stained kidney sections were analyzed for glomerular abnormalities induced by nephritis. Abnormal glomeruli were found at a frequency of 16% in the saline group and 14.9% in the PHPMA group (Figure 1C, 1D). There was no significant difference between these two groups ($P = 0.9$). The frequency of abnormal glomeruli in Dex and P-Dex treated mice was 11.3% and 9.9%, respectively (Figure 1C, 1D). There was no significant difference between the Dex and P-Dex groups ($P = 0.7$), but the frequency in both groups was significantly lower than that in the saline group ($P < 0.01$). Although the frequency of abnormal glomeruli in the Dex and P-Dex groups was lower than that in the PHPMA group, the difference achieved significance for the P-Dex ($P < 5\times10^{-3}$) but not the Dex ($P = 0.07$) group. Thus, both Dex and P-Dex preserve the structural integrity of glomeruli, suggesting that these treatments attenuate nephritis.

P-Dex does not reduce anti-dsDNA IgG levels or renal immune complexes

Nephritis in (NZB×NZW)F1 mice correlates with serum levels of pathogenic anti-dsDNA IgG [10]. Therefore, serum anti-dsDNA IgG levels were assessed. Over the 8-week time course, serum anti-dsDNA IgG levels increased in the saline ($P = 0.07$) and PHPMA ($P < 0.01$) groups, although this increase fell short of statistical significance in the saline group (Figure 2A). Over this time period, serum anti-dsDNA IgG levels rose significantly in the Dex and P-Dex groups (Figure 2A; $P \leq 0.01$), indicating that neither treatment prevented this pathognomonic increase in anti-dsDNA IgG. Nonetheless, at the 8-week time point, serum anti-dsDNA IgG levels in the Dex group were significantly lower than

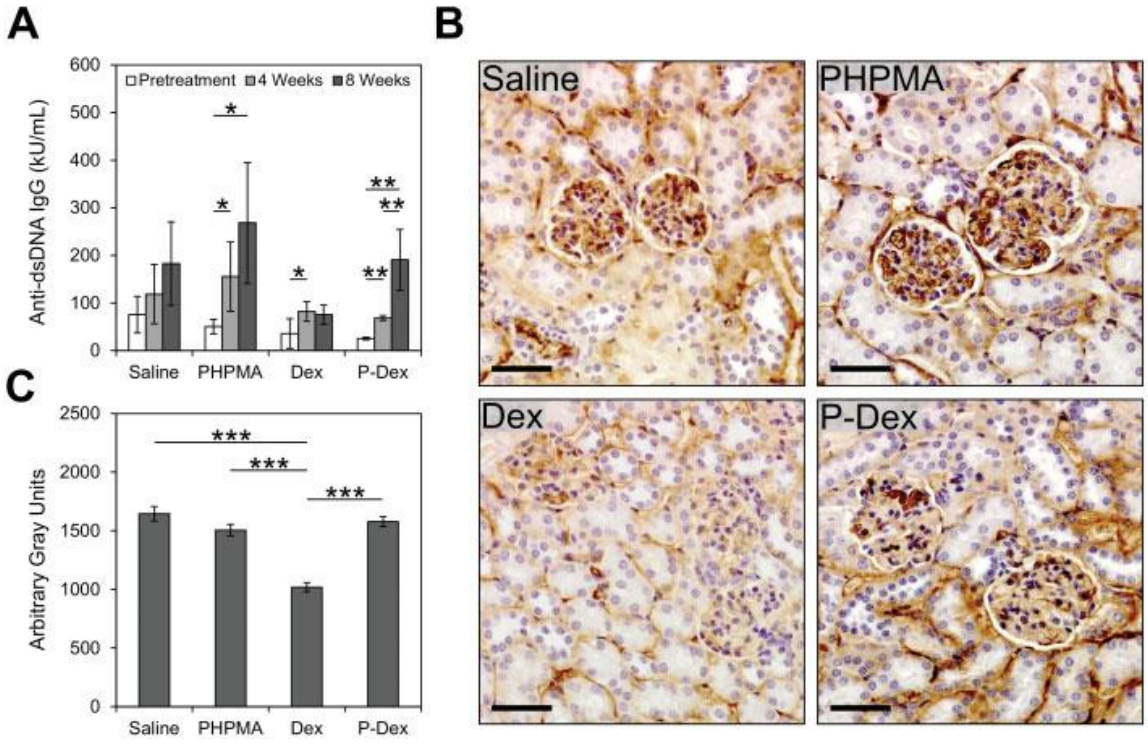

Figure 2. **Effect of different treatments on serum anti–double-stranded DNA (anti dsDNA) IgG and renal immune complex levels.** A, Anti-dsDNA IgG levels at the pretreatment, 4-week, and 8-week time points, as determined by enzyme-linked immunosorbent assay. B, Representative kidney sections from each treatment group, immunohistochemically stained for renal deposition of anti-dsDNA IgG. Bars = 50 μm. C, Quantification of immune complex staining. Values in A and C are the mean ± SEM. * = $P < 0.02$; ** = $P < 0.001$; *** = $P < 1 \times 10^{-12}$. See Figure 1 for other definitions.

those in the PHPMA and P-Dex groups ($P < 0.05$). Thus, Dex blunts the increase in anti-dsDNA IgG, which correlated with reduced incidence of albuminuria and nephritis in this group. By contrast, in the P-Dex group, reduced glomerular damage did not correlate with serum anti-dsDNA IgG, suggesting that P-Dex prevents nephritis through a mechanism that is independent of production of pathogenic autoantibodies.

Because nephritis in (NZB×NZW)F1 mice is associated with renal deposition of anti-dsDNA IgG-containing immune complexes [11], renal immune complex deposition was evaluated (Figure 2B). Quantification of staining indicated that immune complex deposition in the PHPMA treated group did not differ from that in the saline group (Figure 2C; $P \geq 0.2$). Renal immune complex staining in the Dex treated group was significantly less than that in the other groups (Figure 2C; $P < 1 \times 10^{-12}$). Immune complex staining in the P-Dex group was not reduced compared to saline or PHPMA groups (Figure 2C; $P \geq 0.7$). Thus, in contrast to Dex, P-Dex does not prevent nephritis by diminishing renal immune complex deposition.

P-Dex reduces renal macrophage infiltration

To test the hypothesis that P-Dex reduced renal inflammation, we examined renal macrophage infiltration, a marker of chronic inflammation. Staining with the macrophage marker Iba-1 was detected in all groups. Quantification of Iba-1 staining indicated that macrophage infiltration in the saline and PHPMA groups did not differ (Figure 3; $P = 0.4$). Macrophage infiltration was not significantly reduced in the Dex group compared to either the saline ($P = 0.2$) or PHPMA ($P = 0.7$) group (Figure 3). By contrast, renal macrophage infiltration was significantly lower in the P-Dex group than in the saline and PHPMA groups (Figure 3; $P < 0.04$). Although macrophage infiltration was less abundant in the P-Dex group than the Dex group, this difference fell just short of statistical

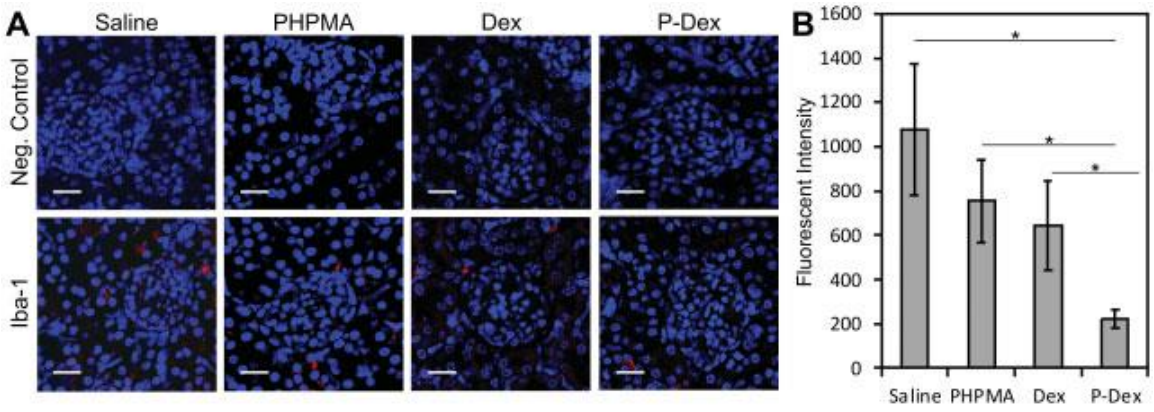

Figure 3. **Impact of treatment on renal macrophage infiltration in (NZB x NZW)F1 mice.** A, Representative confocal microscopic images of immunohistochemical staining of kidney sections from mice in each treatment group. Sections were stained with an anti-Iba1 antibody (red) and DAPI (blue). Negative control (no Iba1 antibody) and merged images are shown. Bars = 25 μm. B, Quantification of Iba1 staining. Values are the mean ± SEM. * = $P < 0.05$. See Figure 1 for definitions.

significance (Figure 3; $P = 0.06$). These results suggest that P-Dex may maintain renal function by attenuating renal inflammation.

P-Dex reduces mean arterial pressure

Because GC therapy can lead to hypertension, we assessed the impact of each treatment on blood pressure. In mice treated with either saline or PHPMA, MAP was not significantly altered after 8 weeks (Figure 4A; $P \geq 0.4$). In the Dex group, MAP was reduced after 8 weeks of treatment (Figure 4A), although this decrease fell short of statistical significance ($P = 0.08$). By contrast, P-Dex significantly reduced MAP after 8 weeks of treatment (Figure 4A; $P < 1\times10^{-4}$).

P-Dex treatment does not affect bone quality

Long-term GC use is associated with osteoporosis. To understand the impact of P-Dex on the skeleton, the femoral BMD and micro-architecture were evaluated. No significant difference in femoral BMD was observed between the saline and PHPMA groups (Figure 4B). Dex treatment, however, was associated with a significantly lower BMD (Figure 4B; $P < 5\times10^{-7}$). By contrast, BMD in the P-Dex group did not differ from that in the saline and PHPMA groups ($P > 0.05$). No significant differences in trabecular bone volume fraction or number were found between groups. However, in the Dex group, trabecular thickness was significantly lower than that in the other groups (Figure 4C; $P < 0.05$). Thus, in contrast to free Dex, P-Dex did not negatively affect BMD or microarchitecture of the bone.

P-Dex treatment does not reduce serum IgG levels

GC therapy causes immunosuppression and reduces serum IgG [12, 13]. We therefore analyzed the impact of treatment on serum immunoglobulin levels. None of the

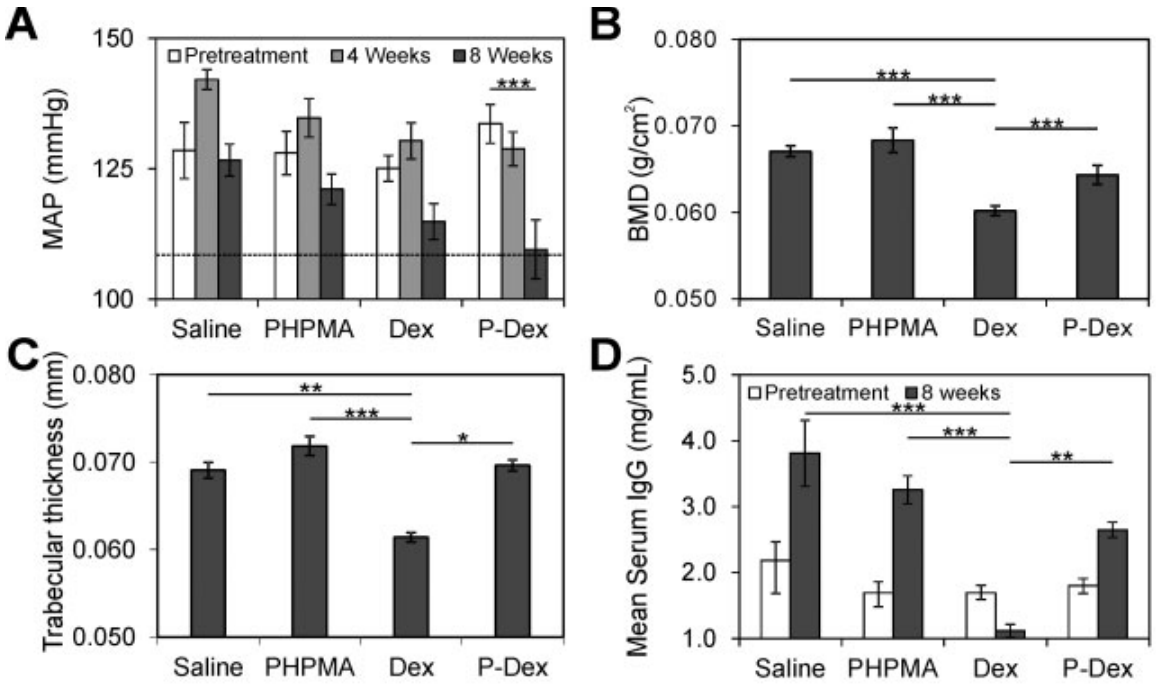

Figure 4. **Analysis of side effects associated with treatment in (NZB x NZW)F1 mice.** A, Mean arterial pressure (MAP) at the pretreatment, 4-week, and 8-week time points, as measured using the tail-cuff method. The horizontal line represents the MAP (mean _ SEM 109 _ 2 mm Hg) in a group of nonautoimmune female NZW mice (n _ 12) ages 4–6 months. B and C, Bone mineral density (BMD) (B) and trabecular thickness (C) after 8 weeks of treatment. D, Total serum IgG levels at the pretreatment and 8-week time points, as determined by enzyme-linked immunosorbent assay. Values are the mean ± SEM. * = $P < 0.05$; ** = $P < 0.001$; *** = $P < 0.0005$. See Figure 1 for other definitions.

treatments affected serum IgA or IgM levels (data not shown). Prior to treatment, no significant differences in serum IgG levels were observed between groups (Figure 4D; $P > 0.4$). In the saline and PHPMA groups, serum IgG concentrations increased significantly after 8 weeks (Figure 4D; $P = 0.01$). In the Dex group, serum IgG level decreased significantly after 8 weeks (Figure 4D; $P < 5\times10^{-3}$). By contrast, serum IgG concentration increased after 8 weeks in the P-Dex group (Figure 4D; $P < 5\times10^{-3}$). At the 8-week time point, Dex treated mice had significantly lower serum IgG levels than mice in the saline, PHPMA, and P-Dex groups ($P < 5\times10^{-3}$). Thus, in contrast to free Dex, P-Dex did not reduce serum IgG.

P-Dex exhibits nephrotropism in (NZB×NZW)F1 mice

To elucidate the mechanism underlying the enhanced efficacy and decreased toxicity of P-Dex in (NZB×NZW)F1 mice, optical imaging was performed to evaluate the *in vivo* distribution of P-Dex. (NZB×NZW)F1 and NZW (healthy control) mice received i.v. injections of P-Dex-IRDye and P-Dex-Alexa and imaging was performed at 2 and 7 d post injection. P-Dex-IRDye preferentially accumulates (2 d) and is retained (7 d) in inflamed kidneys of (NZB×NZW)F1 mice, but not healthy kidneys of NZW controls (Figure 5A). FACS revealed that ~61% of kidney cells from (NZB×NZW)F1 mice were P-Dex-Alexa$^+$ whereas less than 20% of kidney cells from NZW mice were P-Dex-Alexa$^+$ (Figure 5B). Furthermore, the mean fluorescence intensity of P-Dex-Alexa$^+$ kidney cells of (NZB×NZW)F1 mice was substantially greater (~ 4.5 fold) than that of P-Dex-Alexa$^+$ kidney cells in NZW mice. This observation suggests that on a per cell basis, larger quantities of P-Dex are taken up and retained in the kidneys of (NZB×NZW)F1 mice than NZW mice. Persistent near-infrared fluorescence signals were also observed in the spleen and liver of (NZB×NZW)F1 mice (Figure 5A). FACS analysis showed that ~81% of spleen cells and ~10% of liver cells were P-Dex-Alexa$^+$ (Figure 5B). In peripheral

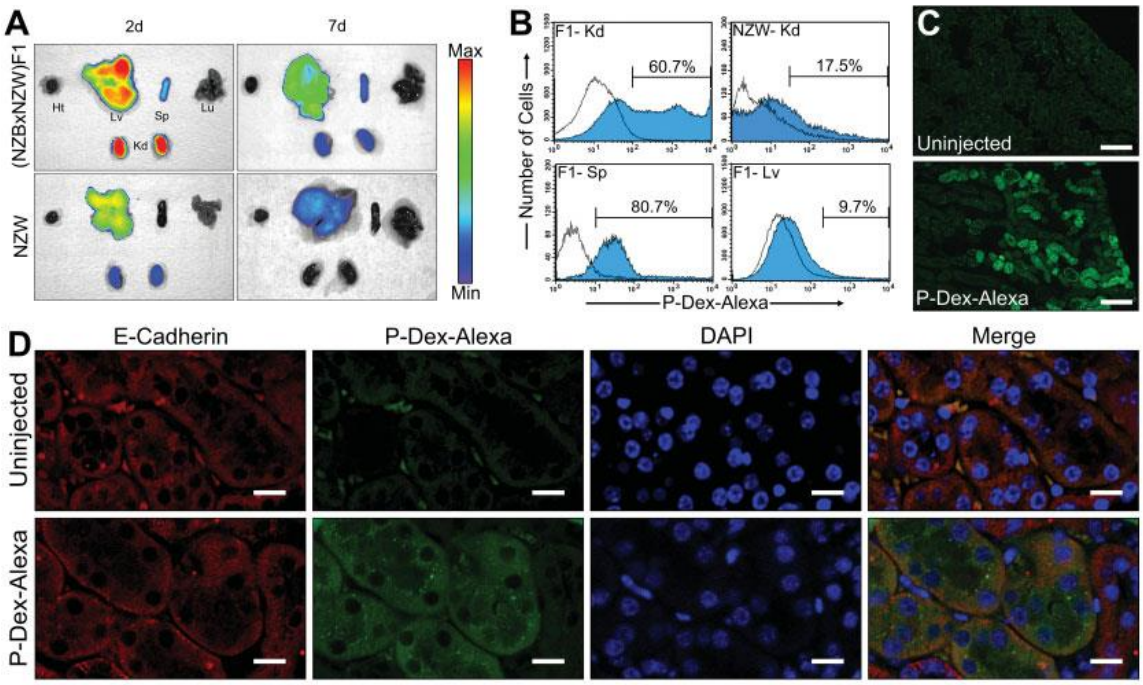

Figure 5. **Nephrotropism and renal cell retention of the dexamethasone prodrug (P-Dex) in (NZB x NZW)F1 mice.** A, Representative optical images of organs isolated from (NZB x NZW)F1 mice and NZW mice. Images were obtained 2 days and 7 days after intravenous injection of IRDye 800CW–labeled P-Dex (P-Dex–IRDye). B, Representative results of fluorescence-activated cell sorting analysis of cells isolated from the organs of (NZB x NZW)F1 or NZW mice without (white) or with Alexa Fluor 488–labeled P-Dex (P-Dex–Alexa) treatment (blue), 7 days after injection. C, Representative fluorescence microscopic images of kidney sections from (NZB x NZW)F1 mice without or with P-Dex–Alexa treatment, 7 days after injection. Bars = 150 μm. D, Representative confocal microscopic images of immunohistochemical staining of kidney sections from (NZB x NZW)F1 mice without or with P-Dex–Alexa treatment, 7 days after injection. Sections were stained with an anti-mouse E-cadherin antibody and DAPI. Antibody signal (red), P-Dex–Alexa signal (green), DAPI signal (blue), and a merged image are shown. Bars = 15 μm. Ht = heart; Lv = liver; Kd = kidney; Sp = spleen; Lu = lung.

blood, ~69.7% of WBCs were P-Dex-Alexa$^+$, but virtually no signal was seen in red blood cells (data not shown).

Renal distribution of P-Dex-Alexa

To identify the mechanism responsible for renal retention of P-Dex, mice were injected with P-Dex-Alexa, and 7 days later, kidneys were isolated and analyzed by fluorescence microscopy and FACS. P-Dex-Alexa$^+$ cells were most abundant in proximal tubules of the renal cortex (Figure 5C). Immunohistochemical staining indicated that a large proportion of retained prodrug was localized within cortical epithelial cells, which were identified based upon histology and E-cadherin positivity (Figure 5D). Flow cytometry indicated that 21.3% of the P-Dex-Alexa$^+$ kidney cells were E-cadherin$^+$ (data not shown). The identity of the remaining P-Dex-Alexa$^+$ cells could not be determined definitively by flow cytometry, despite the use of an extensive panel of antibodies designed to identify various cells in the inflamed kidney. Less than 0.1% of the P-Dex-Alexa$^+$ cells in kidney were F4/80$^+$ macrophages, CD11c$^+$ dendritic cells, CD4$^+$ helper T cells or CD8a$^+$ cytotoxic T cells (data not shown).

Internalization, intracellular localization and activation of P-Dex

Proximal tubule epithelial cells contribute to nephritis by secreting chemokines and inflammatory cytokines in response to albumin and immune complexes [14–17]. Because these cells represent the largest defined population of P-Dex-Alexa$^+$ cells, the internalization kinetics of P-Dex-FITC was examined in HK-2 renal proximal tubule cells *in vitro*. HK-2 cells rapidly internalized P-Dex-FITC (Figure 5A). To test the hypothesis that renal inflammation enhances uptake of P-Dex, we examined prodrug uptake in HK-2 cells treated with LPS, which causes renal inflammation *in vivo* and induces the release

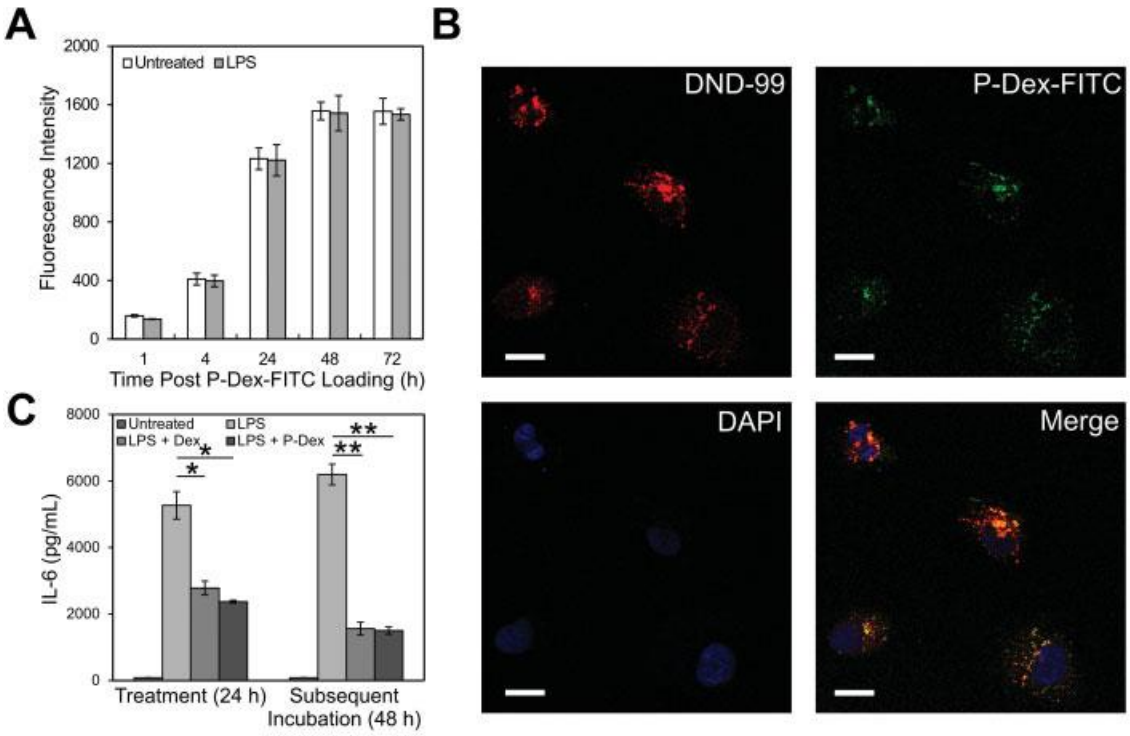

Figure 6. **In vitro internalization, intracellular localization, and activation of fluorescein isothiocyanate (FITC)–labeled dexamethasone prodrug (P-Dex) in human proximal tubule epithelial (HK-2) cells.** A, Quantification of internalized P-Dex–FITC in untreated and lipopolysaccharide (LPS)–stimulated (10 µg/ml) HK-2 cells over a 72-hour time course. B, Representative confocal microscopic images showing internalization and intracellular localization of P-Dex–FITC in LPS-stimulated (10 µg/ml) HK-2 cells. LysoTracker DND-99 signal (red), P-Dex–FITC signal (green), DAPI signal (blue), and a merged image are shown. Bars = 15 µm. C, Impact of dexamethasone 21-phosphate disodium (Dex) and P-Dex on LPS-induced interleukin-6 (IL-6) secretion in HK-2 cells. Values in A and C show the mean ± SEM (n = 3 individual experiments). * = $P < 5 \times 10^{-7}$; ** = $P < 1 \times 10^{-12}$. All treatments (LPS, LPS + Dex, LPS + P-Dex) resulted in a significant increase in IL-6 secretion versus untreated ($P < 1 \times 10^{-4}$). However, asterisks for these comparisons are not shown.

of proinflammatory cytokines and mediators from renal cells *in vitro* [18, 19]. LPS did not alter prodrug internalization kinetics (Figure 6A).

To examine the fate of internalized P-Dex-FITC, we used immunohistochemistry and confocal microscopy. Internalized P-Dex-FITC co-localized with the LysoTracker® lysosome marker in HK-2 cells (Figure 6B), suggesting that P-Dex-FITC is internalized and processed by an endocytic pathway, that results in sequestration in a lysosomal compartment, where P-Dex would gradually undergo processing in the acidic environment, leading to the release of active drug [2–4].

To examine the impact of P-Dex on LPS-induced cytokine release in HK-2 cells, ELISA was used to evaluate secretion of the proinflammatory cytokine IL-6 into the supernatant. Although untreated HK-2 cells secrete low levels of IL-6, secretion can be stimulated 60-fold by LPS (Figure 6C; $P < 1\times10^{-5}$). Dex and P-Dex significantly attenuated the LPS-induced increase in IL-6 secretion (Figure 6C; $P < 5\times10^{-3}$), indicating that both treatments can inhibit secretion of inflammatory cytokines by activated proximal tubule epithelial cells.

Discussion

Recent lupus drug development strategies have focused on targeting specific molecules and pathways that impact immunologic and proinflammatory processes. Although progress has been made, off-target toxicity due to the inability to manage *in vivo* drug distribution still poses a significant clinical challenge. Targeting inflammation with macromolecular prodrugs is a new nanomedicine-based therapeutic strategy. This approach is based on a mechanism involving **e**xtravasation of macromolecules through

leaky **v**asculature and **i**nflammatory cell-mediated **s**equestration (ELVIS) and has been validated in several inflammatory disease models [2, 4, 20].

Based upon these studies, we hypothesized that a dexamethasone prodrug could selectively target lupus-associated renal inflammation and become activated locally to ameliorate nephritis. Furthermore, we postulated that this prodrug would avoid the off-target toxicity associated with traditional GC therapy. To test this hypothesis, we treated (NZB×NZW)F1 mice with saline, PHPMA, Dex, or P-Dex beginning at 4 months of age, prior to the onset of albuminuria. Over the next 8 weeks, the majority of mice treated with saline or PHPMA developed albuminuria. As expected, Dex decreased the incidence of albuminuria by ~50%, and reduced both serum anti-dsDNA IgG levels and renal immune complexes [21]. Strikingly, P-Dex was more effective than Dex and completely prevented albuminuria. P-Dex did not affect serum anti-dsDNA IgG levels or renal immune complexes, but did reduce renal macrophage infiltration. These observations suggest that P-Dex may attenuate nephritis by reducing renal inflammation and act via different mechanisms than free Dex. Importantly, our results also demonstrate that P-Dex does not cause osteoporosis, a major systemic side effect associated with GC treatment.

Based upon the above data and the ELVIS mechanism, we hypothesized that P-Dex would extravasate and be retained at sites of renal inflammation. Optical imaging, immunohistochemistry and FACS analysis confirmed preferential accumulation and retention of P-Dex in inflamed kidneys of lupus-prone but not healthy control mice. Furthermore, these *in vivo* data validate that cell sequestration is the major mechanism for retention of P-Dex in the inflamed kidney, with proximal tubule epithelial cells being the primary cellular reservoir of sequestered P-Dex.

Our data, together with previously published work, support a model in which reduced glomerular capillary macromolecular permselectivity associated with renal inflammation enhances passage of P-Dex into the ultrafiltrate. This in turn leads to uptake and sequestration of significant quantities of the prodrug by the activated proximal tubule epithelium. The glomerulus is responsible for forming a nearly protein-free plasma ultrafiltrate. The essential components of the glomerular filtration barrier, the barrier between the blood and urinary space, are the fenestrated capillary endothelium, the surrounding basement membrane, and the filtration slit (spanned by the slit diaphragm) between adjacent foot processes of podocytes. In the normal kidney, small solutes readily pass through the filtration barrier, with passage increasingly restricted as molecular weights exceed 15 kDa; solutes of >50–60 kDa have very limited passage into the ultrafiltrate. Macromolecules having a net negative charge are further impeded from crossing the filtration barrier. Given that P-Dex is a neutral molecule of ~36 kDa, some P-Dex filtration is likely under normal conditions. Loss of integrity of the filtration barrier results in enhanced permeability (reduced permselectivity) of large molecules such as albumin, leading to albuminuria. Nephritis in (NZB×NZW)F1 mice is associated with vascular damage, alterations in the glomerular basement membrane and distortion of slit diaphragms [22–24], and this likely accounts for the enhanced filtration of P-Dex in these mice. The higher proportion of plasma P-Dex likely passing into the ultrafiltrate in (NZB×NZW)F1 mice compared to controls, results in increased P-Dex delivery to the apical aspect of the renal tubular epithelium. Proximal tubule epithelial cells reabsorb multiple substances from the tubular fluid, and previous reports indicate that polymer carriers can achieve renal targeting through uptake by these cells [25–29]. Enhanced prodrug delivery to and uptake by proximal tubule epithelial cells likely contributes to the efficacy of P-Dex.

Albumin and immune complexes activate proximal tubule epithelial cells and induce secretion of chemokines and cytokines such as IL-6 that promote renal inflammation and immune cell infiltration [14–17]. Our *in vitro* data indicate that P-Dex, similar to free Dex, inhibits LPS-induced IL-6 release from proximal tubule epithelial cells, suggesting that P-Dex may reduce renal inflammation by attenuating the pro-inflammatory response of proximal tubule epithelial cells. We postulated that inflammation would enhance endocytosis and P-Dex uptake in proximal tubule epithelial cells. Though not supported by our cell culture studies, this hypothesis was consistent with results from our *in vivo* studies, which indicate that kidney cells in the (NZB×NZW)F1 mice take up and retain larger quantities of P-Dex than those in NZW mice.

Although proximal tubule epithelial cells were identified as a major population of P-Dex-Alexa^{+} cells in kidney, the identity of a substantial percentage of P-Dex-Alexa^{+} cells in kidney remains to be determined. Furthermore, P-Dex was also found in liver, spleen and peripheral WBCs of (NZB×NZW)F1 mice, which is consistent with our findings in other disease models [2, 4, 30]. A clear understanding of how P-Dex uptake or retention in these cells or tissues impacts the ability of P-Dex to prevent nephritis and reduce off-target toxicities needs further investigation.

In summary, monthly administration of P-Dex provided superior prevention of lupus nephritis and reduced toxicity in (NZB×NZW)F1 mice, as compared to dose equivalent, daily administered Dex. We speculate that the nephrotropism and retention of P-Dex in (NZB×NZW)F1 mice is, at least partially, attributed to reduced glomerular capillary permselectivity and enhanced uptake by activated kidney cells including proximal tubule epithelial cells. The intracellular processing of P-Dex into free dexamethasone and sustained release of active drug at the site of inflammation provides a rational explanation for the superior, sustained anti-inflammatory effect of P-Dex in the local

environment. These data provide a rationale for the future development of this macromolecular prodrug system as a potential preventive and/or therapeutic agent for lupus patients. Further clarification of the mechanisms underlying P-Dex action will be essential for its structural optimization and clinical translation.

Acknowledgments

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Appendix B: A Dexamethasone Prodrug Reduces the Renal Macrophage Response and Provides Enhanced Resolution of Established Murine Lupus Nephritis

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Competing interests: The authors have read the journal's policy and have the following conflicts: Dong Wang is an inventor of a PCT patent application (WO 2005/097073), which discloses the dexamethasone prodrug development. No other coauthor declares any competing interest. The authors confirm that this potential competing interest does not alter their adherence to the PLOS ONE policies on the sharing of data and materials.

Abstract

We evaluated the ability of a macromolecular prodrug of dexamethasone (P-Dex) to treat lupus nephritis in (NZB × NZW)F1 mice. We also explored the mechanism underlying the anti-inflammatory effects of this prodrug. P-Dex eliminated albuminuria in most (NZB × NZW)F1 mice. Furthermore, P-Dex reduced the incidence of severe nephritis and extended lifespan in these mice. P-Dex treatment also prevented the development of lupus-associated hypertension and vasculitis. Although P-Dex did not reduce serum levels of anti-dsDNA antibodies or glomerular immune complexes, P-Dex reduced macrophage recruitment to the kidney and attenuated tubulointerstitial injury. In contrast to what was observed with free dexamethasone, P-Dex did not induce any deterioration of bone quality. However, P-Dex did lead to reduced peripheral white blood cell counts and adrenal gland atrophy. These results suggest that P-Dex is more effective and less toxic than free dexamethasone for the treatment of lupus nephritis in (NZB × NZW)F1 mice. Furthermore, the data suggest that P-Dex may treat nephritis by attenuating the renal inflammatory response to immune complexes, leading to decreased immune cell infiltration and diminished renal inflammation and injury.

Introduction

Lupus nephritis is a leading cause of morbidity and mortality among lupus patients [1]. Lupus nephritis is associated with inflammation caused by renal deposition of immune complexes containing autoantibodies, particularly IgG autoantibodies recognizing double stranded DNA (anti-dsDNA IgG). If not resolved, renal inflammation can lead to renal injury, dysfunction, and failure.

Lupus nephritis can be effectively treated with glucocorticoids (GCs). However, because long-term GC therapy is required, this treatment frequently is associated with numerous side effects involving the endocrine, cardiovascular, hematopoietic and musculoskeletal systems [2]. These adverse side effects, especially secondary osteoporosis, contribute significantly to morbidity in lupus patients. Nevertheless, because of the lack of alternative therapeutic options, GCs continue to be the mainstay of clinical management of lupus nephritis [3].

In an attempt to reduce GC-associated side effects, we previously employed a nanomedicine-based strategy to modify the pharmacokinetic/biodistribution profile of GCs to enhance drug delivery to the site of inflammation while reducing systemic exposure to the drug. Specifically, we developed a macromolecular prodrug of dexamethasone (P-Dex); P-Dex is taken up preferentially by the proximal tubule epithelial cells in the inflamed kidneys of (NZB × NZW)F1 females, but the prodrug is also found to a much lesser extent in splenocytes and circulating blood cells [4]. We observed that P-Dex prevents the development of nephritis in young lupus-prone (NZB × NZW)F1 female mice without causing osteoporosis, a side effect associated with the equivalent dose of free Dex [4]. Our previous studies also suggest that P-Dex prevents nephritis by attenuating the response of the kidney to immune complex deposition and decreasing the recruitment of infiltrating immune cells to the kidney.

Here, we sought to further explore the therapeutic potential of P-Dex for the treatment of lupus nephritis using a preclinical mouse model. The primary objective of the present study was to determine if P-Dex could effectively treat established nephritis in (NZB × NZW)F1 mice. Additionally, we sought to assess the safety of longer term P-Dex administration and to further explore the potential underlying mechanism of action of this prodrug.

Results

P-Dex reverses established albuminuria, extends survival and reduces incidence of severe nephritis and tubulointerstitial disease in (NZB × NZW)F1 mice

To determine if P-Dex could ameliorate established nephritis, P-Dex was administered monthly to (NZB × NZW)F1 females beginning at ~22 weeks of age, after they had developed nephritis, as evidenced by sustained albuminuria. Treatment was continued for 12 weeks. Two control groups, one receiving dose equivalent daily Dex and the other receiving a monthly dose of saline, were also treated for 12 weeks. Mice were monitored for an additional two weeks after cessation of treatment. Over the entire experimental time course, albuminuria not only persisted in 100% of the mice in the saline treated group, but also increased in severity in most of these mice (93%) (Figure 1A). In the Dex group, albuminuria likewise continued in 100% of the mice. However, albuminuria intensified in just 23% of the Dex treated mice, indicating that Dex treatment could prevent progression of renal dysfunction. By contrast, albuminuria resolved in 78% of the mice in the P-Dex group (Figure 1A). Albuminuria persisted but did not increase in the remaining 22% of mice in this group. The fraction of mice in the P-Dex group that showed resolution of albuminuria was significantly greater than that in the Dex treated group, indicating that P-Dex is more effective than dose equivalent Dex in resolving albuminuria associated with lupus nephritis ($P \leq 1 \times 10^{-6}$).

Prior to the end of the experiment, ~55% of mice in the saline group were euthanized due to severe nephritis (Figure 1B). In the saline treated group, median survival was ~13 weeks after initiation of treatment, which corresponded to ~35 weeks of age. The median survival in this group is similar to what we and others have reported previously for (NZB × NZW)F1 females [5-7]. All mice in the Dex and P-Dex groups survived the entire

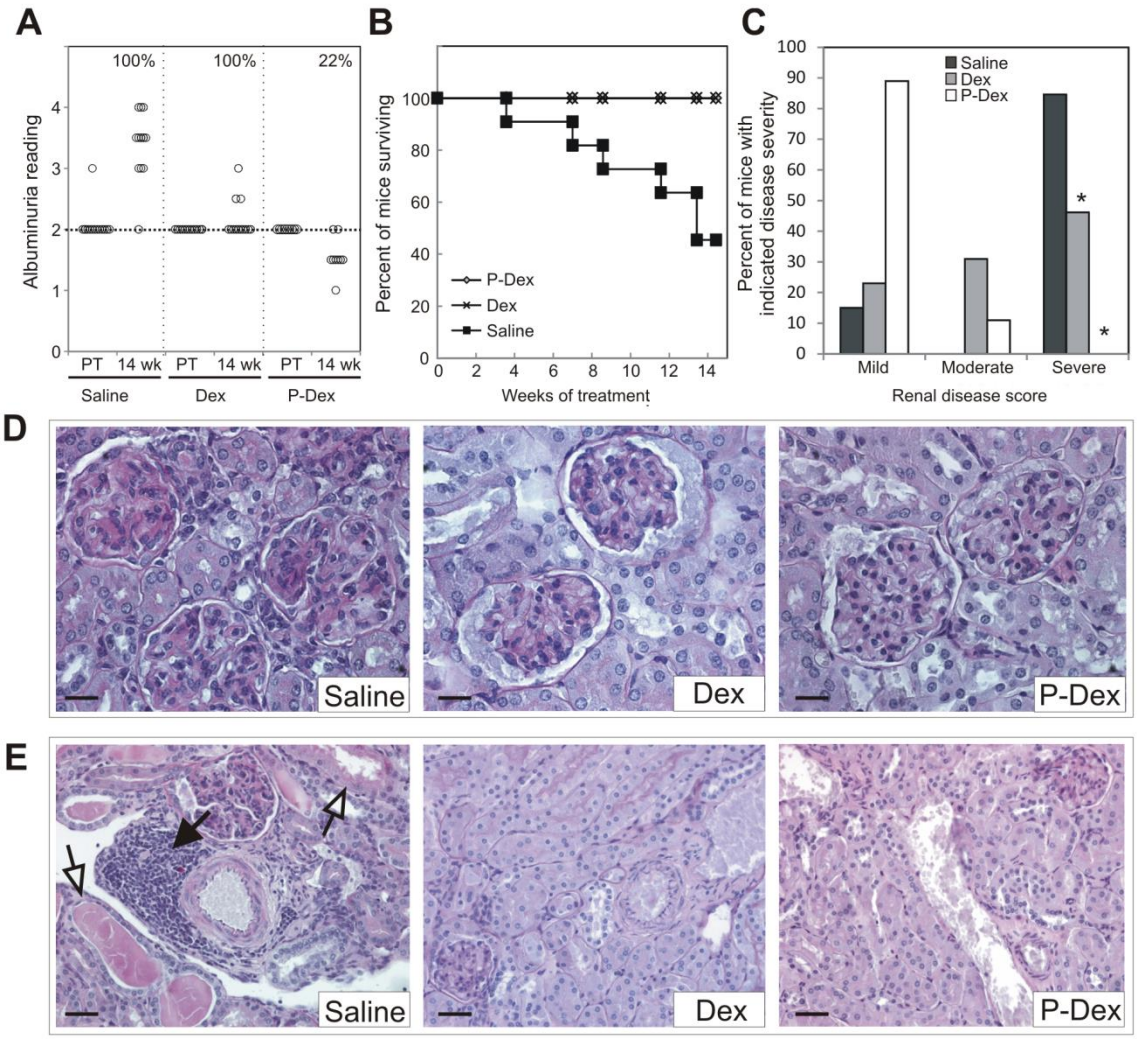

Figure 1. **P-Dex ameliorates albuminuria, extends lifespan and attenuates development of severe nephritis and tubulointerstitial disease in (NZB × NZW)F1 females.**

(A), Albuminuria data for mice in saline (n=13), Dex (n=13), and P-Dex (n=9) treatment groups is illustrated at the pretreatment (PT) and 14-week time points. The incidence of albuminuria at the 14-week time point for each group is shown (in %) in upper right corner of each sub-section. For mice in the saline group that did not survive to the 14-week time point (n=7), the albuminuria reading shown is the last recorded value. (B), A Kaplan-Meier survival curve for each treatment group is shown. (C), The fraction of mice in each treatment group with mild, moderate and severe renal disease is shown. (D), A PAS stained histological section illustrating representative glomeruli from each treatment group are provided. Scale bars: 20 μm. (E), A representative PAS stained histological section illustrating the tubulointerstitium from each treatment group is provided. Scale bars: 40 μm. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group.

treatment period, indicating that both therapies significantly increased the fraction of mice surviving until the end of the treatment period (P = 0.001). These data indicate that Dex and P-Dex can extend the lifespan of (NZB × NZW)F1 mice.

The kidneys from 86% of mice in the saline group showed histological evidence of severe glomerulonephritis, characterized by diffuse glomerular hypercellularity, matrix deposition and crescent formation (Figure 1 C, D). By contrast, in the Dex treated group, the incidence of severe glomerulonephritis was 46%, which was significantly less than that in saline controls (Figure 1C, D; P = 0.04). In the P-Dex treated group, none (0%) of the kidneys showed histological evidence of severe glomerulonephritis; incidence of severe nephritis in this group was different than that in the saline and Dex groups (P <1 $x10^{-3}$).

Furthermore, 100% of the kidneys from mice in the saline group showed evidence of marked tubulointerstitial disease, which was typified by tubular dilation, tubular casts, and immune cell infiltration into the interstitium. The immune cell infiltrates were found in both the cortex and medulla, and were distributed throughout the interstitium as well as in prominent perivascular lymphoid aggregates (Figure 1E). By contrast, in both the Dex and P-Dex groups, there was little indication of tubulointerstitial disease; kidneys displayed mild tubular dilation, sparse tubular casts and scant evidence of interstitial immune cell infiltration (Figure 1E).

P-Dex does not reduce serum anti-dsDNA IgG or glomerular immune complexes

In (NZB × NZW)F1 mice, severity of nephritis typically correlates with serum levels of pathogenic autoantibodies, particularly anti-dsDNA IgG [8]. Therefore, serum anti-dsDNA IgG levels were assessed. In the saline group, serum anti-dsDNA IgG levels increased significantly over the experimental time course (Figure 2A; P = 0.02). By

contrast, in the Dex group, serum anti-dsDNA IgG did not change significantly over the experimental time course (Figure 2A; $P > 0.05$). Consequently, at the end of the experimental time course, serum anti-dsDNA IgG levels in the Dex group were significantly lower than those in the saline group ($P = 0.02$). Serum anti-dsDNA IgG levels increased in the P-Dex group over the experimental time course (Figure 2A; $P = 0.03$). There were no significant differences in serum anti-dsDNA IgG levels between the P-Dex and saline groups ($P > 0.05$). However, serum anti-dsDNA IgG levels in the P-Dex group were significantly greater than those in the Dex group at the end of the experimental time course ($P < 0.008$). These results demonstrate that P-Dex attenuates nephritis through a mechanism independent of the production of pathogenic autoantibodies.

Although our results indicate that P-Dex did not impact the total levels of serum anti-dsDNA IgG, this observation does not preclude the possibility that P-Dex alters the relative abundance of different subclasses of anti-dsDNA IgG autoantibodies. Such an effect could be important given the fact that anti-dsDNA IgG autoantibodies of different subclasses are not equally pathogenic [8-10]. Therefore, we examined the impact of treatment on the levels of anti-dsDNA IgG of each subclass individually. In the Dex treated group, serum levels of anti-dsDNA IgG_1, IgG_{2a}, IgG_3 and were significantly lower than in the saline control group (Figure 2B; $P < 0.05$). By contrast, the levels of serum anti-dsDNA IgG_1 and IgG_{2a} autoantibodies did not differ between the P-Dex and saline groups (Figure 2B; $P > 0.05$). However, the P-Dex treated group did display lower serum levels of anti-dsDNA IgG_3 autoantibodies compared to the saline group (Figure 2B; $P = 0.03$). There were no differences in serum levels of anti-dsDNA IgG_{2b} autoantibodies between any of the groups (data not shown). These results indicate that P-Dex does not

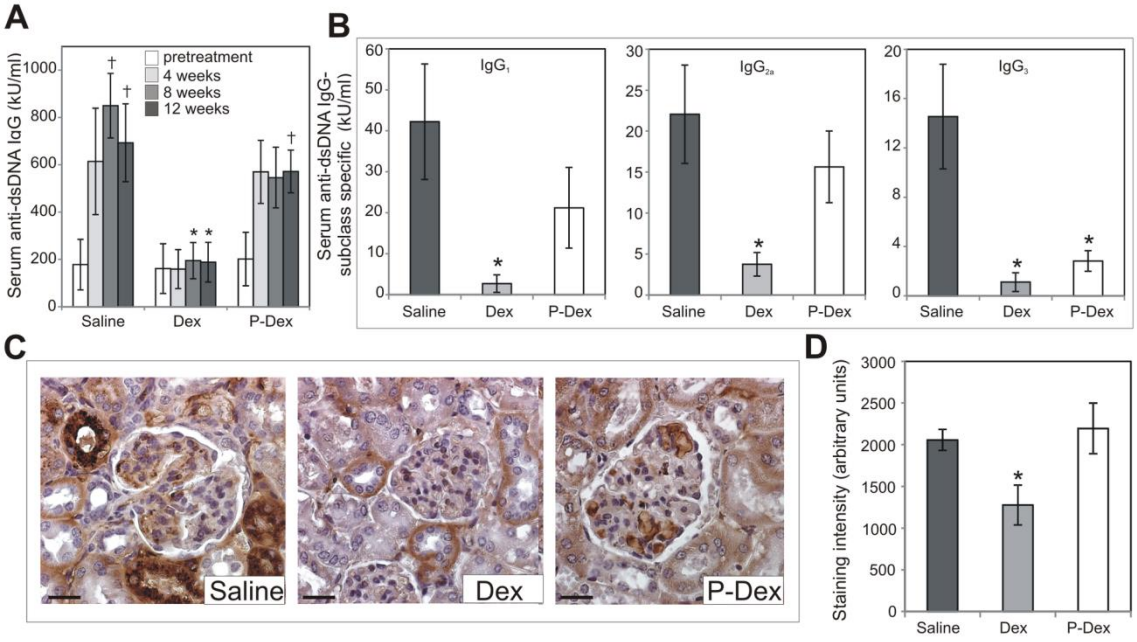

Figure 2. **The effect of treatment on serum anti-dsDNA IgG and renal immune complexes.**

(A), Anti-dsDNA IgG levels for mice in saline (n=13), Dex (n=13), and P-Dex (n=9) treatment groups were determined via ELISA at pretreatment, 4-week, 8-week, and 12 week time points. For the saline group, serum was available for analysis only from the subset of mice surviving at each time point: 12 mice at 4-week time point; 11 mice at 8-week time point; 9 mice at 12-week time point (B), Levels of anti-dsDNA IgG of each subclass were determined via ELISA at the 12-week time point. For the saline group, serum was available for this analysis only from the 9 mice that survived to the 12-week time point (C), Representative sections of kidney from each treatment group are shown. Sections were stained for renal deposition of anti-dsDNA IgG via immunohistochemistry. (D), Quantification of immune complex staining is illustrated. Scale bars: 25 μm; The asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group. The dagger (†) indicates a statistically significant difference ($P < 0.05$) from the pretreatment time point of the same treatment group.

cause a dramatic shift in the relative abundance of different subclasses of anti-dsDNA IgG. Importantly, these results also clearly illustrate that P-Dex does not decrease the abundance of anti-dsDNA IgG_{2a} autoantibodies, which are considered to be the most pathogenic autoantibodies in (NZB x NZW)F1 mice [8-10].

Because glomerular deposition of anti-dsDNA IgG-containing immune complexes contributes to the development and progression of nephritis [11], we evaluated the impact of treatment on glomerular immune complex deposition. In the kidneys of mice in the saline group, prominent glomerular immune complex deposition was observed (Figure 2C, D). In the kidneys of the Dex group, glomerular immune complex deposition was significantly less than that in the saline group (Figure 2C, D; $P = 0.04$). Glomerular immune complex staining in the P-Dex group was similar to that in the saline group (Figure 2C, D; $P > 0.05$), but was significantly different than that in the Dex group ($P \leq 0.025$). Thus, P-Dex treated mice do not develop nephritis despite the presence of abundant glomerular immune complexes.

P-Dex reduces renal macrophage infiltration and tubulointerstitial injury

In (NZB × NZW)F1 mice, the presence of glomerular immune complexes is not sufficient for the development of nephritis. Rather, the development of nephritis requires the recruitment of FcR-expressing myeloid cells, including macrophages, to the kidney and the subsequent activation of these cells by glomerular immune complexes [12,13]. These activated macrophages are thought to contribute to the chronic renal inflammation and tissue damage associated with nephritis. Therefore, the impact of P-Dex treatment on macrophage recruitment in the kidney was evaluated. Quantification of staining with the macrophage marker Iba1 revealed abundant macrophage infiltration into the tubulointerstitium and periglomerular area in the saline group (Figure 3A,B). By contrast,

macrophage infiltration in both the Dex and P-Dex groups was significantly less than that the saline control group (Figure 3A, B; $P < 0.04$). The modest recruitment of macrophages to kidneys from the P-Dex treated mice, despite the presence of abundant glomerular immune complexes, suggests that P-Dex attenuates nephritis by impairing the macrophage infiltration that occurs in response to renal immune complex deposition.

The recruitment of macrophages to the kidney in lupus-prone mice leads to tubulointerstitial inflammation and injury [14,15]. To determine whether the reduced macrophage recruitment in the P-Dex group was also associated with decreased tubulointerstitial injury, the expression of Toll-like receptor 9 (TLR9) and Lipocalin 2 (LCN2), markers for renal tubule damage and tubulointerstitial injury, were assessed [16-20]. Consistent with the observation that the kidneys of the mice in the saline group contained numerous macrophages and showed pronounced tubulointerstitial disease, abundant tubular TLR9 staining was observed in this group (Figure 4A, B). By contrast, in both the Dex and P-Dex groups, there was significantly less tubular TLR9 staining than in the saline group (Figure 4A,B; $P < 0.05$). To assess the impact of Dex and P-Dex on TLR9 expression in a more quantitative fashion, quantitative RT-PCR was performed to determine the level of expression of the *Tlr9* transcript. This analysis confirmed that the levels of *Tlr9* transcript were significantly less in the Dex and P-Dex groups compared to that in the saline control group (Figure 4C; $P \leq 0.01$). Likewise, there was robust expression of LCN2 in the kidneys of the saline group, and significantly less LCN2 in the kidneys of both the Dex and P-Dex groups (Figure 4D; $P \leq 0.05$).

P-Dex prevents the development of lupus-associated hypertension, splenomegaly and vasculopathy

Since systemic inflammation and renal dysfunction promote hypertension in lupus patients and (NZB × NZW)F1 mice [21,22], we assessed the impact of treatment on

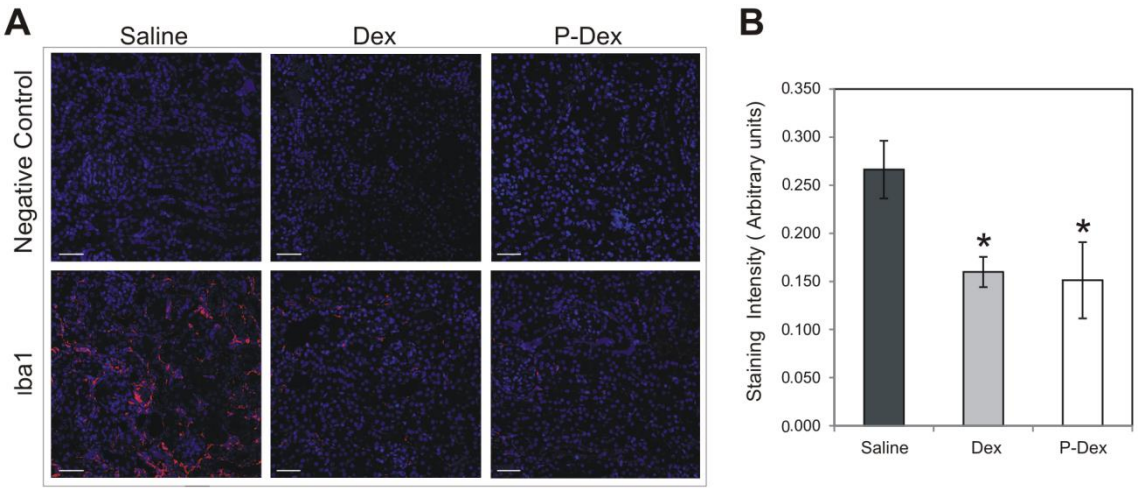

Figure 3. **Impact of treatment on renal macrophage infiltration in (NZB × NZW)F1 mice.**

(A), Representative confocal images of immunohistochemical staining of kidney sections from mice in the saline (n=13), Dex (n=13), and P-Dex (n=9) treatment groups are shown. Sections were stained with an anti-Iba1 antibody (red) and DAPI (blue). Negative control (no Iba1 antibody) and merged images are shown. (B), Quantification of Iba1 staining is illustrated. Scale bars: 50 μm; the asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group.

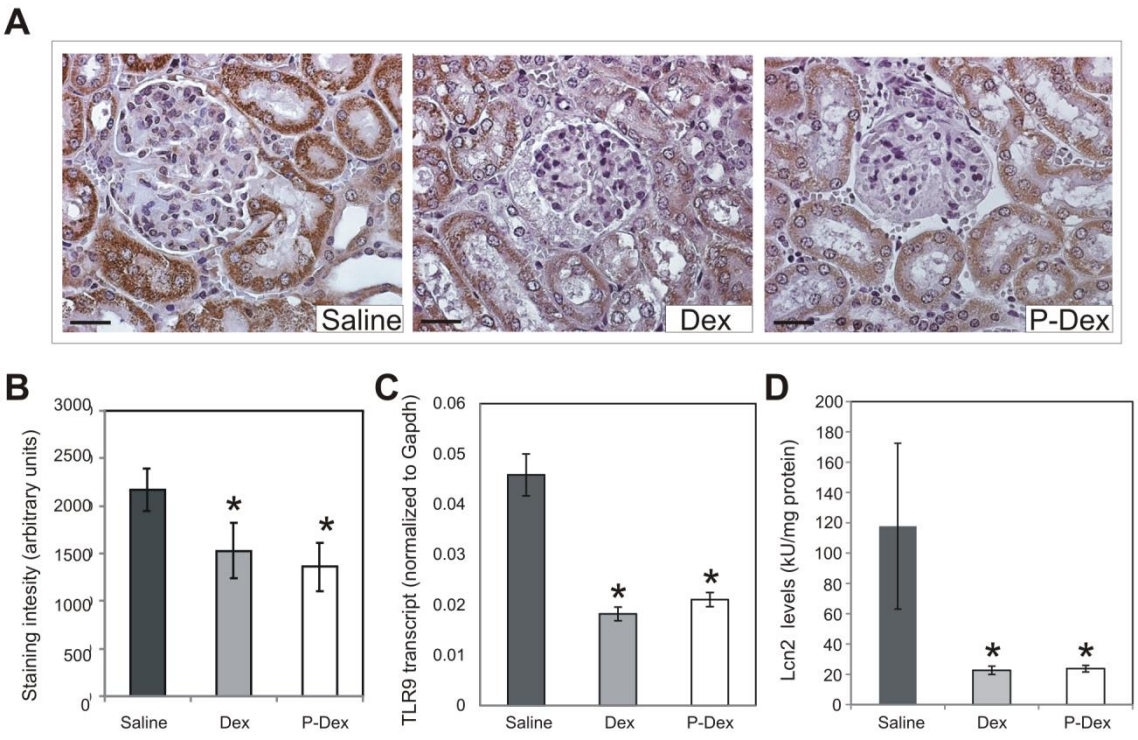

Figure 4**. Impact of treatment on tubulointerstitial inflammation and injury in (NZB × NZW)F1 mice.**

(A), Representative images of immunohistochemical staining of kidney sections from mice in the saline (n=13), Dex (n=13), and P-Dex (n=9) treatment groups are shown. Sections were stained with an anti-TLR9 antibody (brown) and counterstained with hematoxylin (blue). (B), Quantification of TLR9 staining is illustrated. (C), TLR9 transcript levels in the kidney were measured by quantitative RT-PCR. For the saline group, frozen kidneys were available for RNA extraction only from the 6 mice that survived until the 14-week time point. (D), Levels of LCN2 were measured in kidney lysates by ELISA. For the saline group, frozen kidneys were available for preparation of protein lysates only from the 6 mice that survived until the 14-week time point. Scale bars: 25 μm; the asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group.

mean arterial pressure (MAP). Prior to treatment, mice in the saline group were normotensive (Figure 4A). However, over the experimental time course, MAP rose significantly and virtually all of the mice in this group became hypertensive (Figure 5A; $P \leq 0.01$). By contrast, there was no significant change in MAP in either the Dex or P-Dex groups over this time course (Figure 5A; $P > 0.05$).

Splenomegaly occurs only in a subset of human lupus patients. By contrast, splenomegaly is observed in virtually all lupus prone mouse strains, including the (NZB × NZW)F1 hybrid. Therefore, spleen mass of the animals was investigated at necropsy. Splenomegaly was pronounced in the saline group (Figure 5B). By contrast, there was little evidence of splenomegaly in either the Dex or P-Dex treated groups; mean spleen mass in the Dex and P-Dex groups was significantly different than that in the saline group ($P < 0.001$). The spleen mass in the P-Dex group was significantly different than that in the Dex group ($P = 0.03$), although the biological significance of this difference is unclear. All differences persisted when spleen mass was normalized to total body mass (data not shown). Thus, both Dex and P-Dex can attenuate splenomegaly in (NZB × NZW)F1 mice.

Lupus patients are also at high risk for vasculitis. Vasculitis, evidenced by fibrinoid necrosis in the walls of the splenic blood vessels, was found in 54% of mice from the saline group. Fibrin deposits were also noted within the lumen of splenic vessels in 39% of the mice in the saline group (Figure 5C). No fibrin deposition or fibrinoid necrosis was observed in mice from the Dex and P-Dex groups, indicating that both treatments attenuated vascular disease in lupus prone mice.

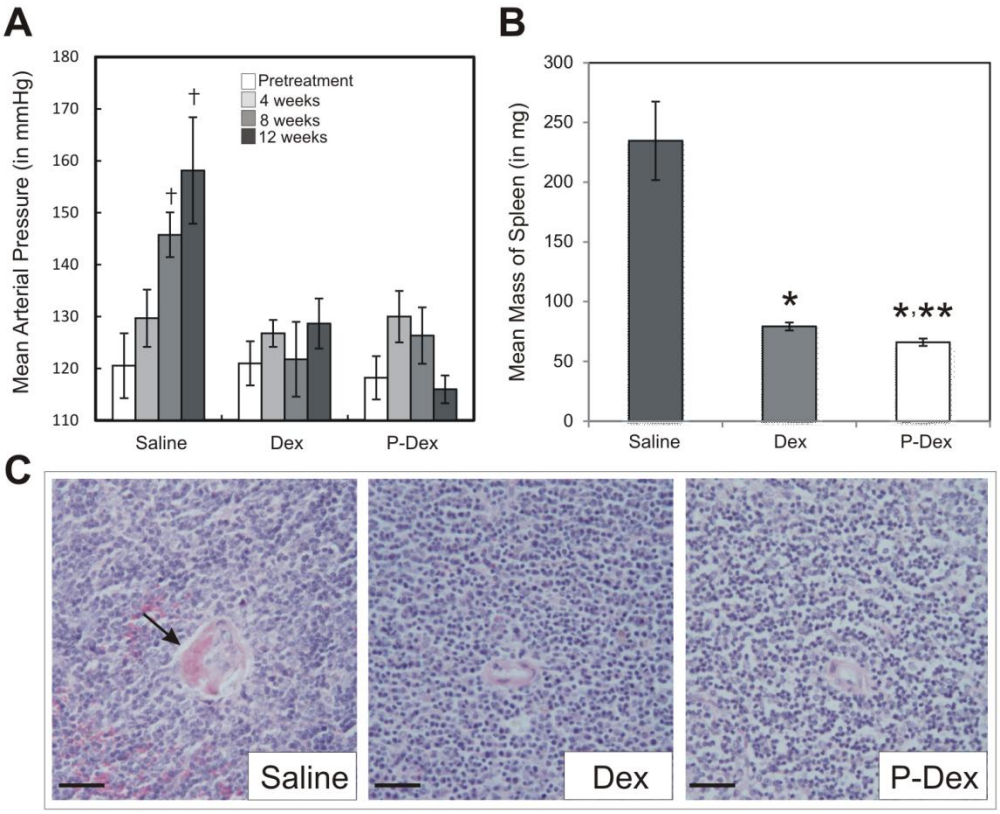

Figure 5. **Impact of treatment on hypertension, splenomegaly and vasculitis in (NZB × NZW)F1 mice.**

(A), Mean arterial pressure was measured at pretreatment, 4-week, 8-week, 12 week time points via tail-cuff method. For the saline group, measurements were obtained only for the subset of mice surviving at each time point: 12 mice at 4-week time point; 11 mice at 8-week time point; 9 mice at 12-week time point (B), Spleen mass was determined at the time of sacrifice in each mouse. (C), A representative hematoxylin and eosin stained histological section illustrating a splenic vessel from each treatment group is provided. The arrow indicates perivascular fibrin deposits indicative of vasculitis. Scale bars: 50 μm; the asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group. The double asterisk (**) indicates a statistically significant difference ($P < 0.05$) from the Dex group. The dagger (†) indicates a statistically significant difference ($P < 0.05$) from the pretreatment time point of the same treatment group. For saline and Dex treatments, n=13; for P-Dex treatment, n=9.

P-Dex treatment does not affect bone quality

Osteoporosis is a major adverse side effect of long-term use of GCs [23]. To investigate the impact of P-Dex on the skeleton, the femoral BMD and micro-architecture were evaluated. As expected, the mean bone mineral density (BMD) and trabecular bone volume/tissue volume (BV/TV) in the femurs of Dex treated mice were significantly lower than that observed in the saline group (Figure 6A, B; $P < 0.05$). Trabecular number did not differ significantly between the Dex and saline groups (Figure 6C; $P > 0.05$). In the P-Dex group, mean femoral BMD, BV/TV and trabecular number did not differ significantly from the means in the saline group (Figure 6A, B, C; $P > 0.05$). By contrast, compared to the Dex group, the P-Dex group exhibited significantly greater BMD (Figure 6A; $P = 0.004$), BV/TV (Figure 6B; $P = 0.007$) and trabecular number (Figure 6C; $P = 0.01$). Thus, unlike Dex, P-Dex did not negatively affect BMD or microarchitecture of the bone.

P-Dex treatment reduces peripheral white blood cells but does not reduce serum IgG levels

GC therapy is associated with immunosuppression [24,25]. Therefore, we monitored peripheral white blood cell (WBC) counts and serum IgG levels during the experimental time course. Prior to treatment, no significant differences in peripheral WBC counts were observed between groups (Figure 6D; $P > 0.05$). In the saline group, peripheral WBC counts initially remained constant, but were significantly reduced at the 12 week time point (Figure 6D; $P = 0.049$). By contrast, peripheral WBC counts were significantly reduced by the 4-week time point in the Dex (Figure 6D; $P = 0.0004$) and P-Dex (Figure 6D; $P = 0.0007$) groups; WBC counts remained low in both of these treatment groups for the duration of the study. No significant changes in serum IgG levels were observed in

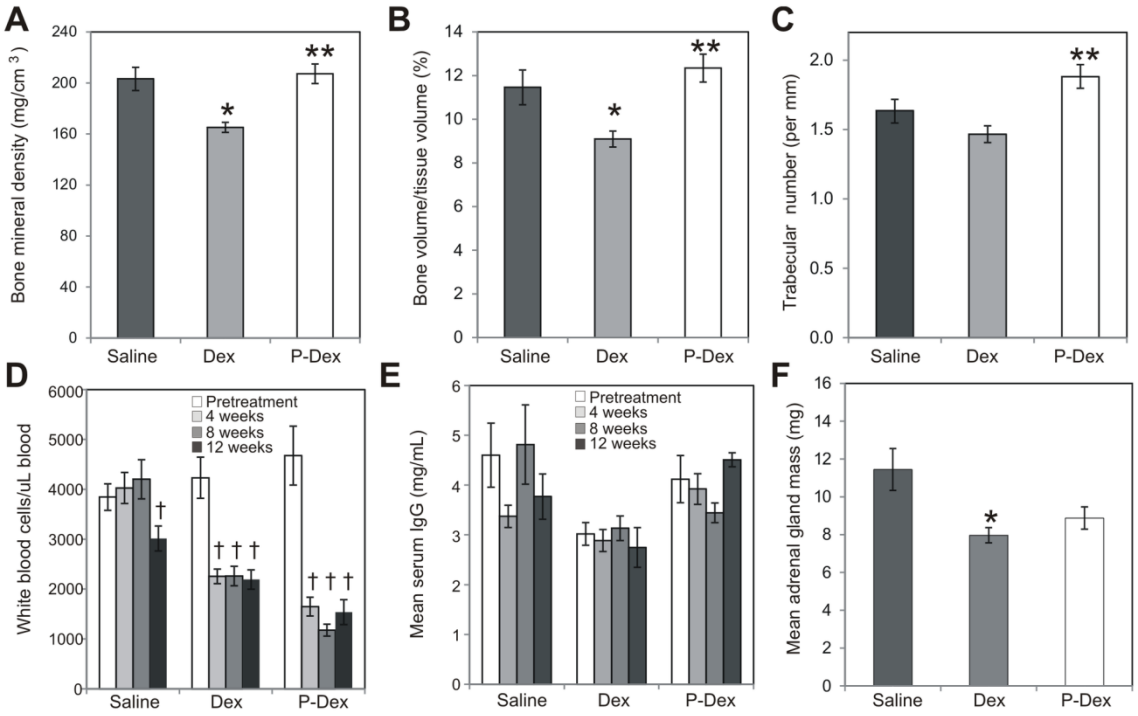

Figure 6. **Evaluation of treatment-induced side effects.**

At sacrifice, femurs were collected for endpoint analysis of bone quality. (A), bone mineral density (B), bone volume/tissue volume and (C), trabecular number measurements in each treatment group are shown. (D), white blood cell counts and (E), total serum IgG levels were determined at pretreatment 4-week, 8-week and 12-week time points. For the saline group, measurements were obtained only for the subset of mice surviving at each time point: 12 mice at 4-week time point; 11 mice at 8-week time point; 9 mice at 12-week time point (F), Adrenal mass was determined at the time of sacrifice in each mouse. The asterisk (*) indicates a statistically significant difference ($P < 0.05$) from the saline control group. The double asterisk (**) indicates a statistically significant difference ($P < 0.05$) from the Dex group. The dagger (†) indicates a statistically significant difference ($P < 0.05$) from the pretreatment time point of the same treatment group. For saline and Dex treatments, n=13; for P-Dex treatment, n=9.

any group (Figure 6E; P > 0.05). Thus, treatment with either Dex or P-Dex reduced peripheral WBC counts, but did not reduce serum IgG levels.

P-Dex treatment induces adrenal gland atrophy

GC therapy causes suppression of hypothalamic-pituitary-adrenal (HPA) axis and atrophy of the adrenal glands. Therefore, at necropsy, we determined the mass of the adrenal glands in each mouse. The mean adrenal mass in the Dex group was significantly different than the saline group (Figure 6F; P = 0.01). Although the mean adrenal mass in the P-Dex group was less than that in the saline group, this difference fell short of statistical significance (Figure 6F; P = 0.07). There was no significant difference in adrenal gland mass between the P-Dex and Dex groups (Figure 6F; P >0.05). These data suggest that treatment with either Dex or P-Dex induced adrenal gland atrophy.

Discussion

Nanomedicine-based approaches that permit the modulation of the *in vivo* pharmacokinetic/biodistribution profile of drugs represent a promising strategy for the development of novel therapeutics to treat lupus nephritis. This approach is particularly helpful for existing drugs, such as glucocorticoids, for which high potency is accompanied by severe side effects due to ubiquitous distribution within the body.

Previously, we demonstrated that P-Dex can be passively targeted to the kidneys of lupus-prone mice, likely due to the leaky vasculature in this inflamed tissue. In the kidneys of lupus-prone mice, P-Dex is internalized and activated by proximal tubule epithelial cells. In our previous study, we found that P-Dex is more effective than free Dex at preventing nephritis in young (NZB × NZW)F1 mice, but it does not cause typical Dex-associated side effects, such as osteopenia [4].

In the present study, we evaluated the potential of P-Dex to treat established nephritis in (NZB × NZW)F1 mice. Over the experimental time course, albuminuria intensified in the mice in the saline control group, and almost all of these mice developed severe nephritis. Furthermore, 55% of the saline treated mice succumbed to severe nephritis. By contrast, all the animals treated with either Dex or P-Dex survived the entire 14-week experimental time course. Although albuminuria worsened in only a fraction of the mice in the Dex group, albuminuria persisted in all of the mice in this group. Strikingly, albuminuria was eliminated in almost 80% of the mice in the P-Dex group. These data indicate the P-Dex improved renal function whereas the equivalent dose of free Dex only maintained the extant level of renal function.

Consistent with our previous observations, we found that P-Dex did not attenuate nephritis by reducing serum anti-dsDNA IgG or glomerular immune complex deposition. We also determined that P-Dex did not attenuate nephritis by causing a shift toward less pathogenic subclasses of anti-dsDNA IgG. However, P-Dex did reduce the infiltration of macrophages into the kidney. The modest recruitment of macrophages to the kidney in the P-Dex group, despite the presence of abundant glomerular immune complexes, suggests that P-Dex may impair the renal pro-inflammatory response to immune complex deposition. It has been suggested that stimulation of the TLR and FcR signaling pathways may act synergistically to initiate the chronic inflammation that leads to nephritis [26]. Both of these pathways are activated by the presence of immune complexes containing dsDNA. Our observation that P-Dex inhibits both tubulointerstitial TLR9 expression as well as the recruitment of FcR-bearing macrophages to the tubulointerstitium, despite the presence of such immune complexes, suggests that the ability to inhibit both of these pathways may contribute to the enhanced therapeutic benefit of P-Dex.

P-Dex also attenuated tubulointerstitial injury and disease. In lupus patients, tubulointerstitial inflammation and injury correlate with impaired renal function more strongly than glomerular damage [27]. Furthermore, tubulointerstitial inflammation is the best predictor of risk of progression to renal failure in lupus nephritis patients [27,28]. Altogether, these data suggest that P-Dex may restore renal function and extend lifespan in (NZB × NZW)F1 females by reducing inflammatory cell infiltration in the kidney, thereby minimizing tubulointerstitial inflammation and protecting the tubulointerstitium from injury. By contrast, Dex inhibits nephritis and extends lifespan in lupus prone mice by reducing serum levels of pathogenic anti-dsDNA IgG autoantibodies.

In our previous study, we found that P-Dex was taken up not only by cells in the kidney, but also by spleen cells in (NZB × NZW)F1 mice [4]. Therefore, in the present study, we evaluated the impact of treatment on the spleen. Neither Dex nor P-Dex induced histopathological abnormalities in spleen. Rather, both treatments attenuated the splenomegaly that develops in (NZB × NZW)F1 mice. Dex and P-Dex also prevented the development of vasculitis affecting the splenic blood vessels.

Due to the passive-targeting of P-Dex to the inflamed kidney, one would expect P-Dex to exhibit a superior safety profile compared to free Dex. To assess the side effects of P-Dex, we measured femoral bone quality, serum IgG levels, peripheral WBC counts and adrenal gland mass. All of these parameters are usually reflective of GC-associated toxicities (e.g. osteoporosis, adrenal gland atrophy and immunosuppression). As expected, Dex treatment significantly reduced femoral BMD and other micro-architecture parameters. By contrast, femoral BMD, trabecular BV/TV and trabecular number in the P-Dex group was not different than that in the saline group. Mice in the Dex and P-Dex groups displayed similar reductions in peripheral WBC counts and adrenal gland

atrophy. This residual toxicity in the P-Dex group is likely due to the fact that P-Dex is taken up to some degree by splenocytes and circulating blood cells [4]. Additionally, these side effects could be due to the free dexamethasone that is released as a result of the cleavage of P-Dex. Collectively, these data suggest that P-Dex treatment partially eliminates the side effects associated with free Dex treatment. Further study is needed to understand why the skeleton was shielded from GC-associated toxicity following P-Dex treatment whereas other tissues and organs remained vulnerable. Acquisition of such knowledge may provide insight that would facilitate the further optimization of the design of this prodrug to improve its safety profile.

Materials and Methods

Ethics statement

Mice were housed under controlled humidity, temperature and lighting conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care, operating in accordance with standards set by the Guide for the Care and Use of Laboratory Animals (The National Academies Press, 1996). Mice were given Harlan irradiated rodent diet 7904 (Harlan Teklad, Madison, WI) and allowed to feed *ad libitum*. All procedures involving live animals were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee under protocol 03-008.

Experimental animals and drug treatment

Beginning at 20 weeks of age, (NZB × NZW)F1 female mice (Jackson Laboratories, Bar Harbor, ME) groups of mice were randomized into saline, Dex and P-Dex groups and monitored weekly for albuminuria using Albustix (Siemens Corp., Washington DC). Albustix readings between 1 and 2 (30-99mg/dl) are considered “normal”, whereas readings of ≥2+ (≥100mg/dl) indicate the presence of albuminuria. Only the mice in each

group with established nephritis, evidenced by sustained albuminuria (≥100 mg/dl) over an initial monitoring period of 3 weeks, were officially enrolled in the study and are described here. The P-Dex (250 mg/kg, containing 30 mg/kg of dexamethasone) and saline groups were administered monthly i.v. injections. The third group was given daily i.p. injections of dexamethasone 21-phosphate disodium (Dex, 1.32 mg/kg, containing 1.00 mg/kg of dexamethasone, Hawkins, Inc., Minneapolis, MN). Over the three-month (12 weeks) treatment period, the overall dose of dexamethasone in the P-Dex and Dex groups was the same. P-Dex was synthesized as described previously [4,29].

Every month, serum was isolated from peripheral blood, mean arterial pressure was recorded via tail-cuff method using the CODA blood pressure measuring system and software (Kent Scientific, Torrington, CT), and peripheral white blood cells were isolated from whole blood and counted by hemocytometer. Mice were weighed and monitored for albuminuria on a weekly basis. Visual inspection of mice showing evidence of increasing albuminuria and/or weight loss was performed daily. Mice that developed severe albuminuria (≥ 2000 mg/dl) or showed signs of distress (i.e. reduced mobility, weight loss >20%, edema, unkempt appearance) were sacrificed immediately. The remaining mice were sacrificed two weeks after cessation of treatment (14 weeks after initiation of treatment). All mice were sacrificed by CO_2 asphyxiation, and tissues were harvested after sacrifice. No anesthesia or analgesia was used.

Analysis of nephritis, renal immune complexes and renal macrophage infiltration

Kidneys were fixed, paraffin-embedded, sectioned and stained with Periodic Acid-Schiff (PAS) (Sigma-Aldrich, St. Louis, MO) and analyzed by light microscopy. Nephritis was assessed using a semi-quantitative 0 to 4 scale as described previously [5].

Renal immune complexes were visualized by immunohistochemistry as described previously [4]. Staining intensity (represented as arbitrary gray units or AGU) of fifty glomeruli per mouse was quantified using region of interest analysis in Axiovision software (v4.6.3.0; Carl Zeiss, Thornwood, NY).

Renal macrophage infiltration was assessed via immunofluorescence with the macrophage marker Iba1 (Biocare Medical, Concord, CA) as described previously [4,30]. Staining was visualized and quantified using confocal microscopy and Zen 2010 software (v6; Carl Zeiss).

Analysis of serum immunoglobulin and autoantibody levels

Serum immunoglobulin concentrations were determined by ELISA (Southern Biotech, Birmingham, AL) as described previously [4,5]. The IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 levels were added together to obtain total serum IgG levels. Serum anti-dsDNA IgG levels were determined by ELISA (Alpha Diagnostics International, San Antonio, TX) as described previously [4,5].

Analysis of markers of tubulointerstitial activation and injury

Renal expression of LCN2 protein was assessed in diluted kidney cell lysates by ELISA (BioPortoDiagnostics, Gentofte, Denmark) according to the manufacturers' instructions. The protein in the supernatant was quantified using the Bradford method. LCN2 expression levels were normalized to total protein input levels.

TLR9 protein expression was assessed via immunohistochemical staining with an antibody specific for this receptor (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously [4,19]. Staining intensity (in AGU) of fifty glomeruli per mouse was quantified using Axiovision software. *Tlr9* transcript level was assayed by quantitative

RT-PCR. For this analysis, total RNA was isolated from kidney using the Absolutely RNA Miniprep Kit (Agilent Technologies, La Jolla, CA) and cDNA using SS VILO Master Mix (Life Technologies, Carlsbad, CA). PCR was performed using *Tlr9*–specific primers [19], SYBR Green PCR Master Mix (Life Technologies) and the Applied Biosystems 7500 Real-Time PCR System.

Histological analysis of vasculitis

Spleens were fixed, paraffin-embedded, sectioned and stained with hematoxylin and eosin stain (H&E) (Sigma-Aldrich, St. Louis, MO) and analyzed by light microscopy using sections from age-matched NZW female mice as a healthy control.

Analysis of bone quality

Femoral BMD and micro-architectural parameters were measured using Skyscan 1172 micro-CT system (Skyscan, Kontich, Belgium) as described previously [4,31]. Micro-CT scanning parameters were identical to those described previously [4]. Femoral BMD, BV/TV and trabecular number and thickness were quantified with CTAn software (Skyscan).

Statistical methods

Comparisons were performed using Fishers exact test, Wilcoxon signed-rank test, Mann-Whitney U test, independent or paired samples t-test, or one-way ANOVA with Tukey's post hoc test where appropriate. Kaplan-Meier survival analysis and log-rank test were used to assess the impact of treatment on lifespan. Statistical analyses were performed using SPSS software (v. 21.0). A two-sided $P \leq 0.05$ was considered significant. Two-sided *P*-values are provided. Mean ± standard error of the mean is presented.

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Author Contributions

Conceived and designed the experiments: FY DW KAG. Performed the experiments: FY DET RKN HY YZ JN KKB KAG. Analyzed the data: FY DET RKN HY YZ KKB SML DW KAG. Contributed reagents/materials/analysis tools: DW KAG. Wrote the manuscript: FY DET RKN DW KAG.

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