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Impact of Estrogen Receptor Alpha Signaling on Plasmacytoid Dendritic Cells in Systemic Lupus Erythematous

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Impact of Estrogen Receptor Alpha Signaling on Plasmacytoid Dendritic Cells in Systemic Lupus Erythematous

by

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Dedication

I would like to dedicate this work to the mice that gave their lives to create it. Research animals suffer through disease and injury to better the health of human and animal kind. Without these animals we would not have the medical tools and knowledge that have saved so many lives.

May all beings be happy and free

May all my thoughts, words, and actions contribute in some way to the happiness of all beings

Acknowledgements

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I would also like to thank my family and friends. My dad encouraged me to go on this long/slightly crazy educational journey. My mom always reminds me of what is really important in life when I think a failed experiment is the end of the world. My brother Chris shares an interest in the medical field and together we enjoy disgusting the rest of the family with medical nonsense. My brother Logan, as a scholar in ancient greek and latin, has helped with my grammar and writing skills. My cat/child Oliver Orange has been my housemate and companion during my time in Charleston. He is a cool cat. Roxana has been a great friend who encourages me to be strong and is the most fun. Eva has also been a great friend and travel buddy. Lastly, I would like to thank Stephen for all the support he has provided the past 3 years. His constant encouragement and belief in me has been critical.

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List of Schemes

List of Abbreviations

AF…… Activation function

B6……. C57BL/6

BM……. Bone marrow

cDC…… Classical dendritic cell

DC……..... Dendritic cell

DHEA……. Dehydroepiandrosterone

ER……... Estrogen receptor

ERαKO…… Estrogen receptor α knock out

FBS…….. Fetal bovine serum

Flt3L……..... FMS-like tyrosine kinase 3 ligand

iDC…….. Inflammatory dendritic cell

IFN…….. Interferon

Il…….. Interleukin

IRF7……. Interferon regulatory factor 7

ISG…….. Interferon signature gene

GM-CSF…….. Granulocyte monocyte colony stimulating factors

GWAS……. Genome wide association study

LDC……. Lymphoid dendritic cell

LN…… Lymph node

mDC……. Myeloid dendrtitic cell

MHC……. Major histocompatibility complex

NK……. Natural killer

NOD…….. Nucleotide-binding oligomerization domain receptors

NZM…….. NZM2410

PBMC……. Peripheral blood mononuclear cells

pDC……. Plasmacytoid dendritic cell

PI3K…… Phosphoinositide 3-kinase

RIG……. Retinoic acid-inducible gene

SLE……. Systemic lupus erythematosus

SLEDAI…….. Systemic lupus erythematosus disease activity index

Spl…… Spleen

TLR……. Toll like receptort

TNF……. Tumor necrosis factor

TREM…….. Triggering receptor expressed on myeloid cells

WT…… Wild type

Abstract

Female lupus prone NZM2410 estrogen receptor alpha (ERα) functionally deficient (ERαKO) mice are protected from renal disease and have prolonged survival compared to wild type (WT) littermates, however the mechanism of protection is unknown. Plasmacytoid dendritic cells (pDCs) and type I interferon (IFN) drive lupus pathogenesis and estrogen acting via ERα enhances both pDC development and IFN production. The objectives for this work were to determine if ERα modulates pDC number, maturation, or function in pre-disease NZM2410 mice as a possible protective mechanism of ERα functional deficiency in lupus prone mice. We measured the effect of ERα functional deficiency on spleen pDC frequency, number, maturation, activation state, and type I IFN activity. ERα functional deficiency reduced the frequency of $MHCII^{+}$ pDCs without altering overall pDC frequency, number, or maturation state. Additionally, ERαKO NZM2410 mice had significantly decreased numbers of pDCs expressing PDC-TREM, a modulator of toll-like receptor (TLR) mediated IFN production. ERαKO NZM2410 mice also had reduced endogenous spleen type I IFN activity. After *in vitro* TLR9 stimulation, ERα functional deficiency significantly reduced the expression of PDC-TREM on pDCs from both NZM2410 and C57BL/6 mice. pDCs from ERαKO mice had reduced levels of PDC-TREM transcripts prior to TLR stimulation, suggesting that ERα signaling alters the pDC's potential to respond to TLR stimulation, thus 'pre-programming' the pDCs. Since PDC-TREM is required for pDC TLR mediated IFN production, this finding represents a mechanism by which ERα impacts pDC IFN production. Thus, we have identified a significant effect of ERα functional deficiency on pDCs and type I IFN activity in pre-disease NZM2410 mice. We believe the modulation of PDC-TREM expression in pDCs

and resultant type IFN activity may represent a mechanism by which ERα functional deficiency protects NZM2410 mice from lupus like disease.

Chapter 1: Rationale and Background

The relationship between the sex bias of systemic lupus erythematosus and dendritic cells

Chapter 1: Rationale and Background

1.1 Rationale

Female ERαKO NZM2410 mice have prolonged survival compared to WT NZM2410 mice [1]. The ERαKO mice survive longer because they do not develop as severe glomerulonephritis, measured by albuminuria and renal pathology [1]. Although the ERαKO mice do not develop clinical kidney disease, autoantibody production and renal immune complex deposition are similar to levels in WT NZM mice [1]. These findings suggested the primary impact of the functional loss of ERα function was on the local tissue response to inflammation or the immune cell response to inflammatory signals (e.g. autoantibodies and immune-complexes).

Dendritic cells (DCs) are activated by immune-complexes and act as a bridge between the innate and adaptive immune responses [2]. DCs consist of two major subsets, the classical DC (cDC) and plasmacytoid DC (pDC). In lupus, DCs (both pDCs and cDCs) impact disease pathogenesis and progression [3, 4]. Mechanistically, dendritic cells (DCs) express Fc receptors, which bind and subsequently internalize immune-complexes [2]. The nuclear material (e.g. DNA and RNA) in lupus autoantibody containing immune-complexes stimulates endosomal toll-like receptors (TLRs) [2]. TLR stimulation results in DC activation and cytokine production, which in turn activates T and B cells in an amplification loop of inflammation [2].

pDCs are of particular interest in SLE because they produce the type I IFNs. Type I IFN signature genes are elevated in patients with SLE and the levels correlate with disease activity [5]. Furthermore, IFN therapy can induce a lupus like syndrome as a side effect. Studies in lupus prone mice have further demonstrated the importance of type I

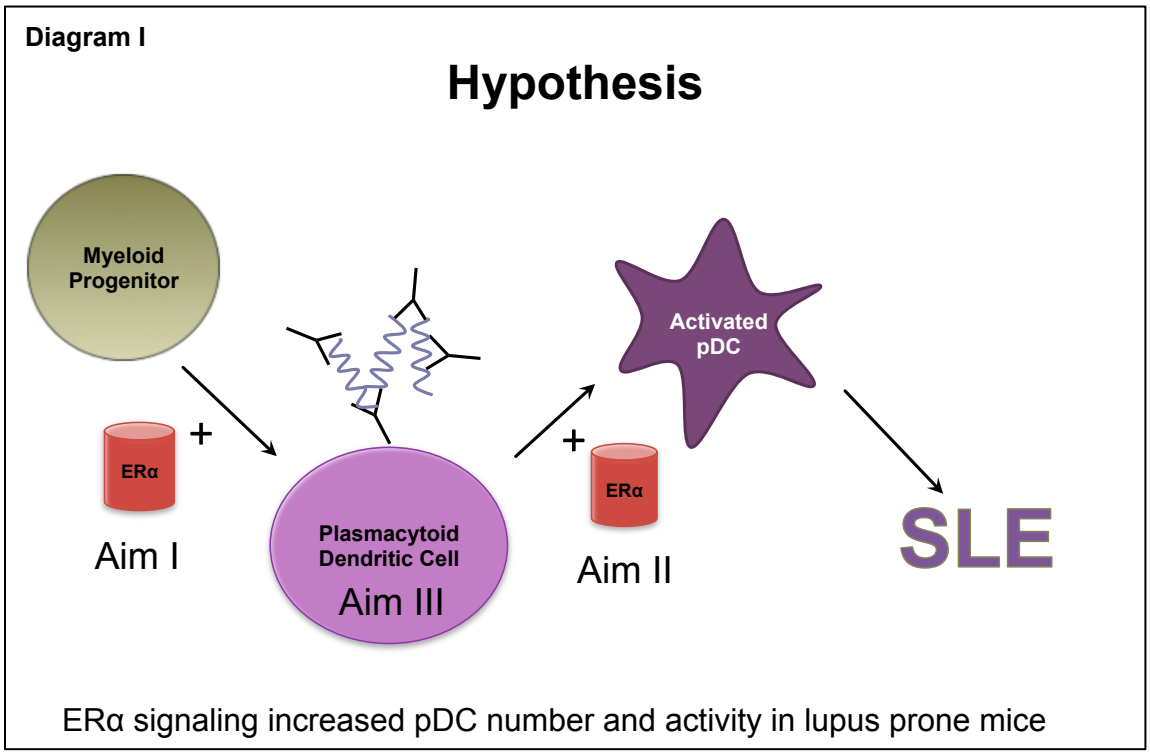
IFNs and pDCs in disease [5]. The absence or blockade of type I IFN ameliorates disease in multiple mouse models [6-8]. Additionally, both partial and transient absence of pDCs prevents clinical disease in mouse models of lupus [9, 10].

In addition to pDCs being central in lupus pathogenesis, estrogen and ERα modulate pDC development and function [11-14]. Estrogen influences DC development and function primarily via ERα [12, 14]. Estrogen via ERα alters both Flt3L and GM-CSF mediated bone marrow derived *in vitro* DC development [11]. We previously published that ERα deficient GM-CSF mediated bone marrow derived DCs, from lupus-prone mice, are less responsive to TLR stimulation *in vitro*, consistent with previous studies in non-autoimmune mice [15]. Regarding type I IFN, long-term exposure to *in vivo* estrogen, via ERα, enhances the type I IFN response of human and murine pDCs in response to TLR stimulation [16]. Given, ERα's role in pDC development and function we are interested in the impact of ERα signaling on pDCs in normal immunity and in SLE.

In this work, we investigated the effect of ERα signaling *in vivo* and *in vitro* on pDC number, development, activation, and function in B6 and NZM2410 mice before and after the development of lupus like disease.

1.2 Hypothesis

I hypothesized that ERα signaling positively impacted pDC number and activation in pre-disease NZM2410 lupus prone mice.



1.3 Background

1.3.1. Lupus Pathogenesis

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by the presence of antinuclear autoantibodies [17]. Although SLE may affect virtually every organ system, symptoms and organ involvement varies widely among individuals. Common symptoms include fatigue, fever, arthritis, skin rash, and renal disease [17]. The prevalence of SLE is 20 to 150 cases per 100,000. However, women account for 90% of cases, making the prevalence rates in women increase to 164 to 406 per 100,000. The exact etiology of disease is unknown and is likely multifactorial [17]. Genetic, environmental, immunologic, and hormonal factors are implicated in disease [17]. Regarding genetics, GWAS studies have identified over 50 genetic loci containing polymorphisms that increase risk for SLE. Of these loci, the polymorphisms in the major histocompatibility (MHC) locus are the most common and increase the risk of SLE most significantly [17]. Environmental factors associated with increased risk for disease include viruses, UV light, silica dust, and medications [17]. SLE patients have numerous immunologic abnormalities and it has proven difficult to separate abnormalities responsible for disease from abnormalities caused by disease. One immunological hallmark of lupus is the production of anti nuclear antibodies, which may occur years prior to onset of clinical symptoms [17]. These antibodies bind to self-nuclear material to form immune complexes. The immune complexes deposit in tissue and activate both immune and stromal cells [18]. For this reason, it is tempting to attribute tissue damage to immune complex deposition. However, the presence of immune complexes is not the complete immunological picture, as many people with anti nuclear

antibodies do not develop clinical symptoms of lupus [17]. Therefore a second level for immune dysregulation must occur to induce clinical disease. This second immune dysregulation may occur in cells of the innate immune system [18]. Innate immune cells contribute directly to tissue injury, release cytokines, and activate autoreactive T and B cells [18]. One mechanism by which the adaptive and innate immune cells communicate in SLE is by innate immune cell uptake of immune complexes. The uptake of immune complexes activates innate immune cells and perpetuates the inflammatory response. Since autoantibody production and immune complex deposition alone are not sufficient to cause disease, it is likely that aberrant activation of the innate immune cells by immune complexes represents the second level of immune dsyregulation required for organ damage.

1.3.2 SLE sex bias

SLE affects women at a nine to one ratio compared to men. Additionally, disease incidence is highest during the reproductive years [19]. Based on this epidemiologic data, the sex hormones and their receptors are thought to play a role in disease.

Sex hormone levels Many studies have examined levels of sex hormones in disease and during flares. These studies have yielded contradictory results. However, a meta-analysis concluded that female SLE patients have reduced DHEA, progesterone, and testosterone and increased estradiol and prolactin [20]. Male patients may have increased prolactin and possibly a reduction in DHEA. Male SLE patient's levels of testosterone and estrogen are normal [20].

Estrogen Receptor expression in SLE In PBMCs from lupus patients, RNA and protein levels of ERα are increased compared to healthy controls [21, 22]. ERβ transcripts were decreased in SLE PBMCs compared to controls [21]. In a study of 22 female lupus patients and 17 controls, T cells from SLE patients had a slight (non significant) decrease in ERα and the same levels of ERβ compared to controls [23]. However, SLE T cells expressed higher levels of calcineurin and CD154 in response to ERα and ERβ agonists compared to controls, suggesting SLE T cells are more responsive to estrogens than healthy T cells [23].

Exogenous estrogen The effects of exogenous estrogens can be divided into two categories. The first category is the effect of exogenous estrogens on disease development and the second is the effect of exogenous estrogens on disease activity.

Development of SLE*:* The effects of estrogen containing oral hormonal contraceptives and hormone replacement therapy have also been controversial in disease. The past use of estrogen containing oral contraceptive was shown to be associated with a significant increase (1.9 fold) of developing lupus [24]. In a later case control study, a rate ratio of 1.19 (95% CI 0.98-1.45) was associated with any use of combined oral contraceptive (estrogen and progesterone) [25]. The greatest risk was found in newly started short term use (<3 months) with a rate ratio of 2.52 (95% CI 1.13-5.57) [25]. Additionally, there was an increased rate of SLE in women receiving high dose of ethinyl estradiol (50ug) compared to women receiving medium (31-49ug) and low (<30) doses of ethinyl estradiol [25].

SLE activity: Regarding the effect of estradiol on SLE activity, the results have also been controversial, however, in a large double-blind prospective study the use of oral contraceptives was found not to increase the risk of flares in women with SLE [26]. Hormone replacement therapy was not shown to increase risk of severe flares, but does significantly increase mild/moderate disease flares [26].

SLE and menstrual cycle

Based on self-reported data, 36% of women with SLE flare prior to menses [27]. Additionally, menstrual cycle disorders are observed in 50% of SLE patients [28].

Selective estrogen receptor modulators; tamoxifen and raloxifene:

In NZB/NZW F1 mice, tamoxifen improves disease [29]. However, in a small (n=11) human double-blind crossover trial, tamoxifen did not improve disease [30]. Regarding raloxifene, in NZB/W F1 mice, raloxifen administration protects mice from estradiol mediated autoreactivity [31]. In a double-blind randomized control trail of menopausal women with SLE, raloxifene improved bone density in SLE patients without increasing SLE activity [32]. In this study, a there was a slight, but non significant, reduction in disease flares and activity in the raloxifene group compared to placebo [32].

Selective estrogen receptor down-regulator; Fulvestrant:

In a double-blind and placebo-controlled study, the effects of fulvestrant were studied 16 premenopausal women with moderate SLE over a 1 year period [33]. In the group

receiving the fulvestrant, patients were able to reduce the dose of prednisone, hydroxychloroquine, and azathioprine compared to the control group [33].

DHEA: DHEA has been studied in randomized controls trials for lupus and has been shown to be moderately beneficial [26, 34]. The use of DHEA, current SERMs, and SERDs are not widely accepted as a viable treatment option due to side effects and lack of large studies.

Mouse models: In lupus mouse models, estrogen treatment accelerated disease and androgen treatment improved disease [35]. Additionally, ovariectomized female mice had delayed onset of disease and the castration and estrogen treatment of male mice caused disease to progress similar to female mice [35]. In the NZB/W mice have a strong sex bias, with the females progressing faster than male nice. The NZM2410 mice also have a sex bias towards the female mice, however, this sex bias is weaker than the NZMB/W background [36]. The absence of functional ERα in female NZM2410 mice reduced kidney disease and increased survival [1]. Protection was not provided by the absence of ERβ [1]. The absence of functional ERα or ERβ in male mice did not provide protection, however, this may be due to the male mice not developing severe disease at the time of sacrifice as there were trends toward decreased disease in ERalpha deficient mice (32 weeks) [1].

1.3.3 Dendritic cell biology

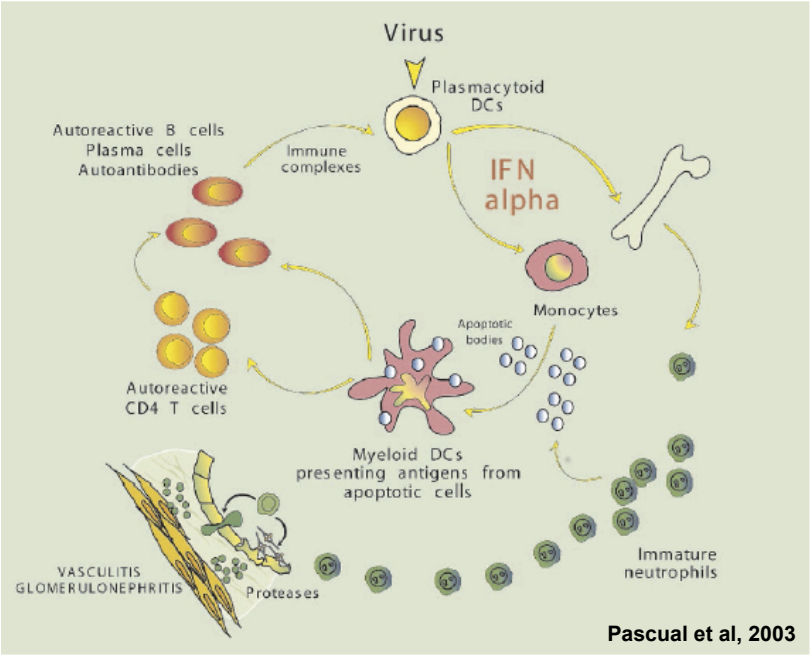
The term dendritic cell (DC) encompasses two types of cells, the plasmacytoid dendritic cell (pDC) and classical dendritic cell (cDC). Briefly, pDCs express endosomal toll-like receptors 7 and 9 (TLRs) [37]. When the TLRs are stimulated by their ligands, typically viral DNA/RNA, pDCs produce large amounts of type I IFNs [37]. The type I IFN function to alert the innate and adaptive immune systems to the presence of infection. Through this mechanism pDCs play an important role in viral immunity. The specifics of pDC biology will be described in the following chapters. Here, we will review the basic functions of cDCs. cDCs are best known as antigen-presenting cells that induce a primary immune response [2]. cDCs endocytose antigens of foreign and self origin and present these antigens to T cells via MHCI or MHCII. cDCs also express T cell co-stimulatory molecules (CD80/86) and produce signal three cytokines (IL-12) [2]. Depending on the environment and quality of the antigen, cDCs can induce a Th1 or Th2 response or immunologic tolerance [2]. Although all cDCs function to present antigen, the term cDC encompasses many subtypes of DCs, each with specialized function. Due to the plasticity of DC populations, DC ontogeny is complex. Most basically, cDCs can be diveded into five categories; **Lymphoid $CD8^+$ DCs, lymphoid $CD11b^+$ DCs, tissue $CD103^+CD11b^-$ DCs, inflammatory $CD11b^+$ DCs, and migratory DCs**. The DC subtypes differ in their expression of pattern recognition receptors, location, and regulators**. Lymphoid $CD8^+$ DCs** live in the lymphoid tissue (spleen) and are best known for sampling the blood for microbial stimuli and presenting antigens (and cross presenting) to $CD8^+$ T cells via MHCI [38]. **Tissue $CD103^+CD11b^-$ DCs** share the MHCI presentation function with the **lymphoid $CD8^+$ DCs**, however they live outside of

the spleen and sample the environment of a specific tissue [35, 38]. **Lymphoid CD11b$^+$** cells also reside in the lymphoid tissue and act as a viral sensor. Upon viral stimulation, they secrete high levels of cytokine and present antigen via MHCII [38]. **Inflammatory CD11b$^+$** cells are thought to arise from both the DC lineage and monocyte lineage [38]. Their maturation is induced by inflammation and are present only during an active inflammatory response [32]. **Migratory DCs** live in tissue and mature and migrate to the draining lymph node upon stimulation with microbial products. They play a role in antigen presentation and tolerance [38].

Dendritic cells in SLE

Dendritic cells are innate immune cells involved in lupus pathogenesis and lupus organ damage. pDCs express Fc receptors, which bind to the Fc regions of autoantibodies in immune complexes [39]. Once the immune complex binds the Fc receptor pDCs endocytosis the

Diagram 2 Dendritic cells in lupus pathogenesis



DCs promotes lupus pathogenesis by producing IFN alpha, presenting self antigens, and activating T and B cells

immune complex. Inside the cells the nucleic acid portion of the immune complex stimulates endosomal TLRs (TLR7/9). TLR stimulation results in activation of the pDC to produce IFNα. The IFNα activates T, B, NK, and classical dendritic cells (cDCs) [39]. cDCs take up apoptotic cells and can inappropriately stimulate autoreactive T and B cells [4]. Additionally, cDCs can function in the organs to facilitate tissue damage [40]. Scheme 1 from Pascual et al. summarizes the role of pDCs and cDCs in lupus pathogenesis [4]. Although we began describing the role of pDCs in lupus pathogenesis, it is unclear what cell type initiates the cycle of inappropriate immune activation. Since lupus pathogenesis involves multiple levels of immune dysregulation, disease is probably initiated/perpetuated by dysfunction in multiple cells types.

DC dysfunction has been noted in lupus prone animals [41]. Additionally, DC dysfunction is noted in human SLE. Although the conclusions from these studies are complicated by differences in DC isolation and culture systems, cDC and pDC abnormalities in cytokine production, activation, and number are present in SLE patients (addressed in chapters 2 and 3). The requirement of DCs for disease progression in mice was shown by the deletion of DCs in multiple lupus prone mouse strains [3, 9, 10]. In MRL/lpr mice, the constitutive deletion of CD11c+ cells (cDCs and pDCs) prevented renal damage [3]. Although the deletion of CD11c+ cells protected the mice from clinical manifestations of lupus (i.e. renal damge), renal immune complex deposition was unchanged in the DC deficient animals compared to controls [3]. Therefore, DCs likely play a role in perpetuating organ damaged caused by immune complexes without hindering the production of autoantibodies or the deposition of immune complexes. Recently, two papers have shown the deletion of pDCs alone ameliorated disease in lupus

prone mice. Both the transient deletion of pDCs and the haplodeficiency of pDCs reduced disease in lupus prone mice [9, 10]. These deletion studies in mice have confirmed the importance of pDCs in lupus pathogenesis.

Plasmacytoid Dendritic Cells in SLE

Plasmacytoid dendritic cells (pDC) impact lupus pathogenesis [42]. In SLE, autoantibodies form immune complexes with self nucleic acids. pDCs take up the immune complexs via Fc Receptors and respond to the self nucleic acids in the immune complexes primarily through toll-like receptors (TLRs) [16]. Once the TLRs are stimulated, pDCs produce inflammatory cytokines, including type I interferon (IFN). pDCs rapidly produce type I interferon (IFN) in quantities 200 to 1,000 times more than other cell types [43]. Other cell types including PMNs also make IFNalpha. Though on a per cell basis, pDCs produce significantly more IFNalpha than PMNs, due to their being significantly more PMNs than pDCs leads to uncertainty regarding the contribution of one cell type vs another in the pathogenesis of lupus. Type I IFNs activate DCs, NK, B and T cells to increase cytokine production and antigen presenting capacity resulting in activation of the body's innate and adaptive immune responses [44, 45]. Type I IFNs can cause loss of self-tolerance as evidenced by development of a reversible drug-induced lupus like disease in patients treated with IFNα for hepatitis C [46]. Type I IFN serum levels are elevated in SLE and correlate with disease activity [46]. Mouse models have solidified the importance of both type I IFNs and pDCs in disease. In lupus prone mice, the expression of an IFN gene signature in myeloid and lymphoid cells precedes disease onset [47]. Additionally, systemic treatment with IFNα of lupus prone mice exacerbated

disease by inducing serve glomerulonephritis [48]. The deletion of the type I IFN receptor protects NZB and NZM2328 lupus prone mice from disease [6, 7]. In the NZM2328 model the disease protection provided by the absence of IFN signaling was correlated with decrease function of cDCs [7]. In BXSB mice, which have a duplication of the TLR7 gene, treatment with an anti-IFN-alpha/beta receptor antibody reduced kidney disease and increased survival [8]. These human and murine studies support the hypothesis that type I IFN signaling plays an important role in SLE. Two IFNα monoclonal antibodies are being tested in SLE patients, sifalimumab and rantalizumab [49]. These antibodies partially reduced IFN signature gene expression in peripheral blood mononuclear cells and skin [49]. Additionally, sifalimumab improved skin and joint symptoms [49]. However, sifalimumab was less effective in reducing the IFN signature gene expression in patients with severe disease (mean SELDAI score of 11) and similar findings were produced with rontalizumab treatment. . In the end, neither of these drugs reached their primary endpoints and were thus "failures". Trial design and dosing may have overshadowed a positive drug effect. The partial effectiveness of these drugs suggest it may be more powerful to more effectively block Type I interferon activity to achieve therapeutic benefit.. However, more complete blockade of type I IFN signaling may impair the ability of the body to flight viral infections such as seen with some kinase inhibitors used in RA. Therefore, to create more effective therapies we must better understand type I IFN signaling in SLE and the mediators of its effect.

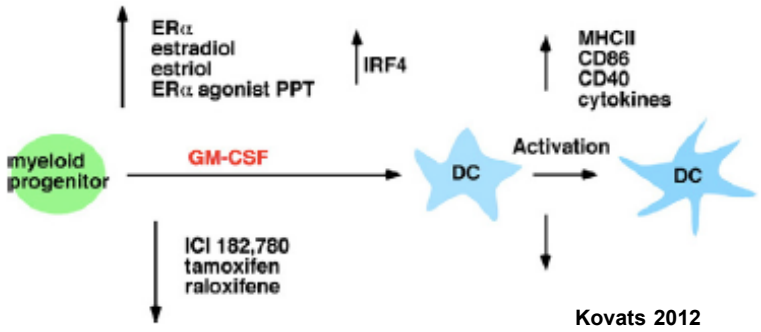
Dendritic Cells and Estrogen

Mouse and human immune cells express estrogen receptors. In mice, mature B and NK cells expression both ERα and β [50]. T cells, pDCs, cDCs, and macrophages express only ERα [50]. In humans, B cells express the highest amount of ERα [50]. pDCs, T cells, NK cells, and monocyte derived DCs express intermediate levels of ERα [50]. Monocytes express the lowest levels of ERα [50]. ERβ is expressed by B cells and pDCs [50].

A few studies have looked at sex differences in expression of estrogen receptors. Monocytes from premenopausal women express less ERα compared to monocytes from men and postmenopausal women [50]. However, no differences have been detected in T cells, B cells, or pDCs [50].

Mouse DCs are obtained by culturing BM with either GM-CSF or Flt3L. Both cytokines are important for DC development *in vivo*. GM-CSF drives DC generation during inflammation and Flt3L generates DCs during the steady state [51]. BM cells are cultured with GM-CSF to obtain a population of $CD11c^{hi}$ $CD11b^{+}$ DCs that resemble the monocyte derived inflammatory DCs (iDC). BM can also be cultured with Flt3L to obtain mDCs, LDCs, and pDCs. The cell populations obtained from the Flt3L culture system represent the steady state DC populations found in the spleen [52]. Both of

Diagram 3 ERα's impact on GMCSF mediated DC development



ERα positively regulates expression of MHCII, CD86, CD40 and inflammatory cytokine production by GMCSF mediated derived DC by promoting IRF4 transcription

these culture systems are regulated by estrogen. Regarding GM-CSF driven BM DC cultures, the addition of estradiol preferentially promotes the generation of $CD11c^{hi}CD11b^{int}$ cells that express high levels of MHCII and CD86 (Scheme 2) [13]. Furthermore, estradiol signaling promotes the generation of fully functional iDCs [12]. The iDCs cultured in the absence of estradiol signaling express less MHCII and CD86 expression after TLR4 stimulation and they produce less inflammatory cytokines upon engagement of CD40 [12] (Scheme 2). The effect of estradiol was specifically found to be through ERα regulation of IRF4 transcription [12-14] (Scheme 2). In contrast to GM-CSF BM cultures, estradiol via ERα reduces DC numbers from Flt3L driven BM DC cultures, suggesting estradiol differently regulates each DC subset [11]. Regarding pDCs, estradiol/ERα signaling increases pDC production of IFNα in response to TLR7/9 stimulation [16, 53]. Although, the majority of studies were carried out in B6 mice, one study has looked into the effect of estradiol on BMDC cultures from lupus prone mice. This study found the addition of estradiol to BMDC cultures from young (predisease) mice reduced BMDC inflammatory responses, however, when estradiol was added to BMDC cultures from mice with clinical disease the estradiol enhanced the inflammatory responses [54]. This study suggests lupus disease activity impacts estradiol's role in DC function. However, more studies are needed to understand estrogen's impact on dendritic cells in the setting of autoimmunity and inflammation. Additionally, studies have almost exclusively studied the role of estrogen in DC development and function using *in vitro* culture and *in vitro* stimulation. Therefore, more works needs to be done to understand how estradiol/ERα alters *in vivo* DC development and function.

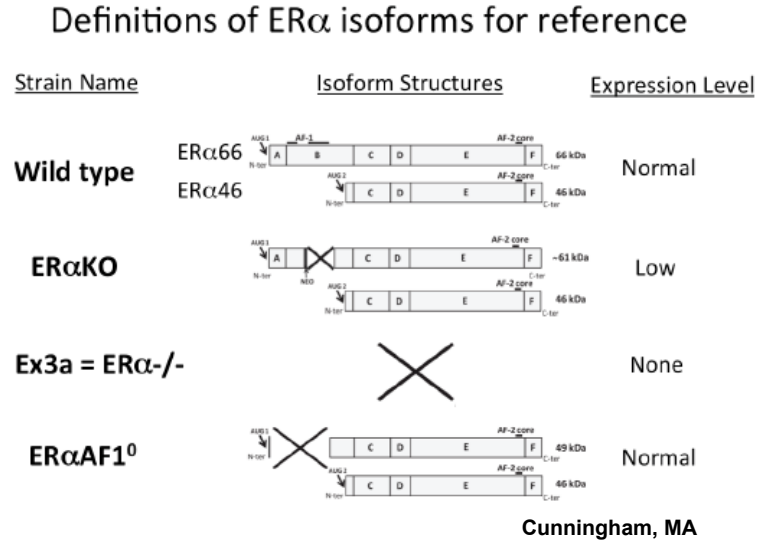
1.3.4 NZM2410 Mice

The NZM2410 mice were created by mating the litter mates from a NZB/WF1 cross with NZW mice [36]. The NZM2410 mice have a weak gender bias towards female mice, produce IgG anti-ds DNA, and die of immune complex mediated glomerulonephritis by 6 months of age [36]. The genetics of the NZM2410 mouse has been studied extensively. 3 lupus susceptibility loci were identified in the NZM mice [36]. These loci have been individually crossed onto B6 mice to study their role in disease. Loci 1, sle1, contributes to the production of autoreactive B and T cells and the production of autoantibodies. Sle2 causes B cell hyperactivity. Sle3 decreases levels of activation-induced cell death of CD4+ cells [36]. Based on these studies, it was determine that the likelihood of developing disease is dependent on the number and balance between susceptibility and resistance alleles [36]. Variations in the murine susceptibility genes have been identified in human SLE. Some of the alleles include complement receptor 2, SLAM family members, and Fcyr2b [36].

1.3.5 Model of ERα deficiency

The ERα 'deficient' mice used for this work and in most other studies of ERα deficiency are not null for ERα [55]. The ERα deficient (ERαKO) mice were produced by inserting a neo cassette into exon 2 and the mouse expresses an N terminal truncated chimeric ERα protein lacking the activation function 1 (AF-1) domain, but retaining the activation function 2 (AF-2), DNA binding, and ligand binding domains [55] (Scheme 3). The truncated ERα protein in the knockout mice resembles both the endogenously expressed ERα46 splice variant and the AF-1 null ERα mutant (Scheme 3). Both ERα46 and AF-1 null isoforms have a functional impact in immune cells, including DCs. Seillet et al. showed that the AF-1 domain is differentially required for DC development [56]. The AF-1 domain is required for steady state DC development and early stages of inflammatory DC development, however, it is dispensable at later stages of inflammatory DC development [56]. Regarding the endogenously expressed ERα46 splice variant, monocyte to macrophage transition induces ERα46's expression, which correlates with MHCII expression on these cells [57]. ERα46 is also expressed by human T, B and NK cells [58]. Based on this evidence it is possible the truncated ERα protein, expressed by ERαKO mice, may have

Diagram 4



function and potentially impact disease as well as the immunological findings presented in this article. As the relative contribution of the lack of an AF1 domain versus the possible function of a truncated ERα are delineated, it is important to understand the mechanisms by which this relative "ERα deficiency" is protective in lupus and impacts DC activity in normal immunity and autoimmunity. Since the ERαKO is not a true deficiency, this model will be referred to as the functionally mutated ERα.

Chapter 2: Impact of estrogen receptor alpha signaling on plasmacytoid dendritic cell number

Chapter 2: Impact of estrogen receptor α signaling on plasmacytoid dendritic cell number

2.1 Introduction

Dendritic cell populations

During the steady state *in vivo* there are three DC populations in mouse lymphoid organs. The populations are pDCs and two subsets of $CD11c^+$ DCs (cDCs). The cDCs are further divided into $CD11b^+$ and $CD8a^+$ cDCs. pDCs produce large amount of type I IFNs in response to viral infection, CD8a+ cDCs present antigen to CD8+ T cells, and CD11b+ cDCs produce inflammatory cytokines and present antigen to CD4+ T cells [38]. Additionally, during the steady state there are tissue resident DCs, which function to present antigen in the lymph node [59]. During the steady state DC differentiation is dependent on Flt3L [52]. Endothelial cells, mast cells, T cells, and bone marrow fibroblasts, and IFNy differentiated macrophages produce Flt3L [60-62]. However, other cellular sources likely exist, particularly during the inflammatory state. In an inflammatory state, GM-CSF drives the development of a different type of DC, the inflammatory DC (iDC) [38, 51]. iDCs develop from GR-1+ monocytes and produce Il-12 and induce the differentiation of antigen-specific Th1 cells [51]. GM-CSF is produced by endothelial cells, fibroblasts, mast cells, macrophages, and T cells [63]. Bacterial endotoxins, IL-6, IL-1 and TNFa can induced GM-CSF production [64]. Flt3L and GM-CSF are used *in vitro* to culture DC populations from BM hematopoietic progenitor cells. Flt3L driven cultures produce lymphoid organ steady state DCs (mDCs/CD11b+, LDCs/CD8a+, and pDCs) and GM-CSF driven cultures produce iDCs [52]. Traditionally Flt3L is thought to be responsible for steady state DC development and survival, as DCs generation is impaired in Flt3L KO mice. However, recent evidence suggests Flt3L also

impacts DC response during infection [65]. Flt3L is produced and released by mast cells in response to infection and the Flt3L release mediates the expansion of the CD8+ cDC subsets [65]. Furthermore Flt3L is increased in the synovial fluid and serum of rheumatoid arthritis patients and the absence of Flt3L protects mice from developing collagen induced arthritis [66, 67]. This finding highlights the complexity of DC regulation *in vivo* and the need to study the DC populations *in vivo* under inflammatory/disease conditions to best understand their function in disease.

Dendritic cells in SLE

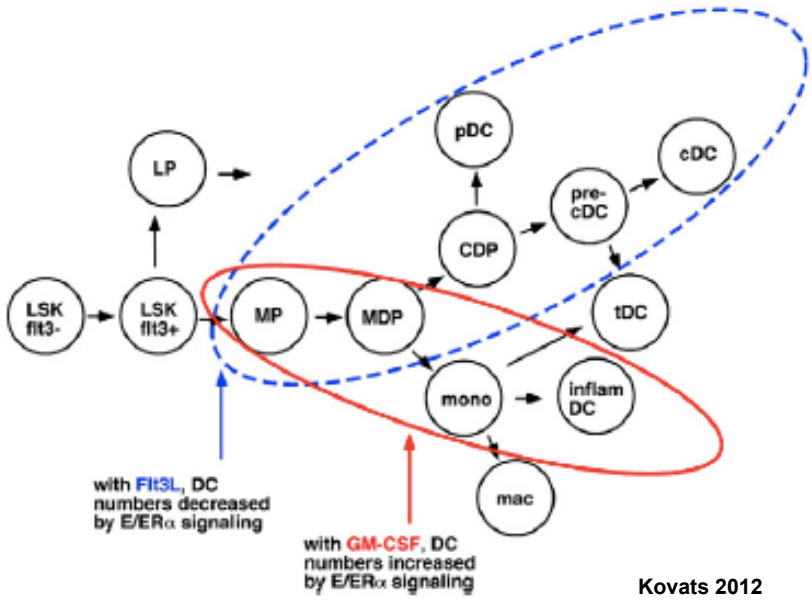
DC populations in SLE patients have been studied, however, the results are inconsistent. The inconsistences are likely due to differences in isolation and culture of the DC subsets as well as confounders found in all studies of patients based on disease severity, disease length and medications as well as individual variability. According to multiple early studies, SLE patients have reduced numbers of blood pDCs. The blood number are likely reduced because the pDCs have infiltrated the effected organs [5]. pDCs are found in both human lupus skin and kidney lesions [5, 68]. However, some studies have identified normal to increased levels of pDCs in the blood from SLE patients [41]. Regarding cDCs, studies have reported low to normal levels of cDCs in the blood of SLE patients [7]. Difficulties in identifying and isolating human DCs confounded these studies. Thus, new technologies and a better understanding of DC biology will help to accurately identify and understand the role of DCs in SLE. In murine lupus, one study reported pDC and cDC frequencies and numbers are increased in the spleen due to increased homing to the spleen during disease [69]. Another study found increased

CD11c+ cells in the spleens of lupus mice [70]. However, this area has not been extensively studied and the markers used in these studies were not specific. Therefore, more work must be done to identify DCs in lupus prone mice.

ERα and DC development

Estrogen via ERα impacts both GM-CSF and Flt3L mediated development of DCs (Scheme 4). Estradiol signaling in GM-CSF driven cultures promotes the development of iDCs by both increasing the number of iDCs generated and the inflammatory potential of the iDCs [12, 13]. *In vivo*, estradiol/ERα signaling also promotes the development of $CD11c^{+}CD11b^{+}$ iDCs [51]. During culture with Flt3L ERα signaling takes on 2 roles. The first role is to reduce numbers of hematopoietic progenitor cells [11]. The second is to promote the development of fully functional DCs [11]. Therefore estradiol decreases the overall number of DCs produced in Flt3L cultures and promotes the function of the

Diagram 5 ERα's impact on GMCSF and Flt3L mediated DC development



Flt3L DC numbers decreased by ERα signaling
GM-CSF DC numbers increased by ERα signaling

remaining DCs. Thus, ERα signaling is important for the number and function of DCs. However, more work is needed to understand how ERα signaling impacts DC populations *in vivo*. Additionally, DC populations should be studied during inflammation, as the role of ERα differs in the inflammatory state compared to the steady state.

Our laboratory found that ERα signaling positively impacts GM-CSF mediated BM cultures from lupus prone mice [15]. The impact was more profound in DCs derived from lupus prone mice compared to B6 control mice. To further understand ERα's role in lupus prone DC development the work presented in this paper investigates ERα's impact on DCs generated from Flt3L cultures from lupus prone mice and pDC populations present *in vivo* both prior to and after the development of clinical disease.

Plasmacytoid dendritic cell development

pDCs develop from common DC progenitors (CDP) and common lymphoid progenitors (CLP) that are $Flt3^{+}$c-Kit^{high}. In addition to pDCs, CDPs also yield cDCs and CLPs yield T, B, and NK cells [37]. No pDC specific progenitor has been identified. Flt3L drives the proliferation and maturation of the common progenitors and the survival of mature pDCs [37]. Interestingly, the pDCs are more sensitive to changes in Flt3L levels compared to cDCs [37]. Flt3L signaling in pDCs has not been studied extensively, but it likely signals through PI3K activation of mTOR [37]. pDCs are also more sensitive to rapamycin (mTOR inhibition) than cDCs. Once pDCs develop to maturity they do not proliferate. pDC life span is poorly understood.

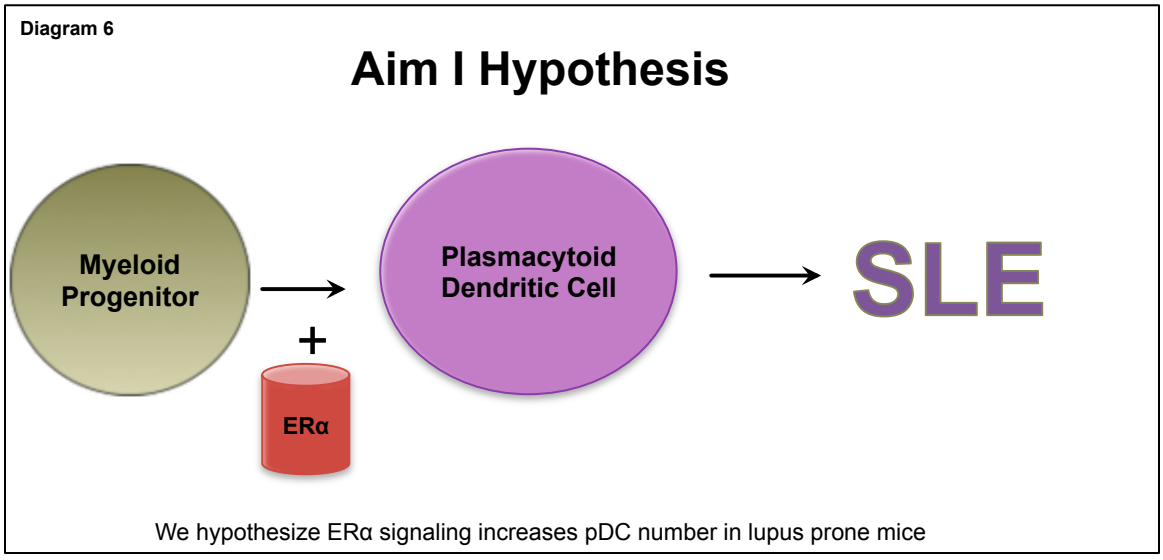
Molecularly, pDC fate is determined by the expression of the basic helix-loop-helix transcription factor E2-2 [71]. pDCs require E2-2 to develop and to maintain their

pDC status [72]. If E2-2 is absent in a mature pDC they will begin to express the cDC transcription factor Id2 and develop a cDC phenotype [72]. Mechanistically, E2-2 promotes the transcription of pDC specific genes and represses expression of cDC specific genes [72].

pDCs develop to maturity in the BM and acquire the surface expression of the pDC specific receptor Ly49Q [73]. Ly49Q expression denotes pDCs as mature in the BM and is required for optimal IFNα production [74]. Once mature, the pDCs exit the BM and populate peripheral lymphoid organs via the blood stream [75]. pDC exit of the bone marrow is dependent on the transcription factor Runx2, which control expression of the chemokine receptors CCR2 and CCR5 [75].

2.2 Hypothesis

Since pDCs impact lupus pathogenesis and ERα signaling alters *in vitro* Flt3L mediated pDCs development in healthy mice, **I hypothesize ERα signaling impacts the frequency, number, activation, and function of pDCs in pre-disease NZM2410 mice.** These changes in pDCs may account for the disease protection of ERα deficiency in ERαKO NZM mice.



2.3 Results

ERα signaling increases plasmacytoid and myeloid DCs in Flt3L driven BM cultures from NZM2410 mice

ERα impacts *in vitro* DC development in Flt3L driven BM cultures from B6 mice, but the effect of ERα on Flt3L driven BM cultures from other strains and specifically lupus-prone mice is not known [11, 56]. To assess the impact of ERα signaling on DC populations in lupus prone mice, we cultured BM from WT and functionally mutated ERα (ERαKO) female NZM2410 (NZM) mice with Flt3L to produce bone marrow derived DCs (BMDCs). We identified the standard BMDC populations of pDC, myeloid DC (mDC), and lymphoid DC (LDC) populations by flow cytometry. We studied post-

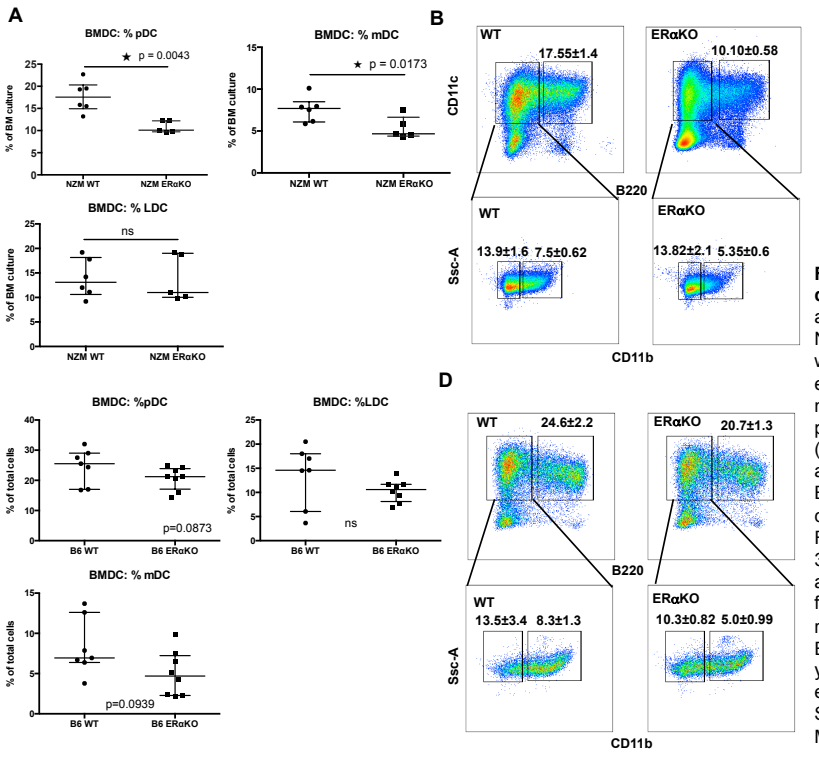


Figure 1. DC populations in Flt3L driven BMDC cultures. BM from WT and ERαKO 10-14 week old female NZM2410 and B6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. The functional mutant of ERα reduced the percent of pDCs ($B220^+CD11c^+$) and mDCs ($CD11c^+$, $B220^-$, $CD11b^+$) without altering the percent of LDCs ($CD11c^+$, $B220^-$,$CD11b^-$) obtained from the BM cultures in both NZM **(A)** and B6 **(C)**. Flow cytometry plots representative of 3 separate experiments; totaling 6 animals per genotype **(B, D)**. Cultures from NZM ERαKO mice generated a reduced cell yield and cultures from B6 ERαKO mice generation increased cell yield compared to WT **(E).** Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

pubertal pre-disease (10-14 week) NZM mice to improve the likelihood of identifying a change in DC populations responsible for disease development rather than a secondary change caused by the disease environment. Only female mice were studied based on our prior description that the functional mutation of ERα provides protection from disease in females [1]. Additionally, we cultured the cells under low estrogen conditions, using charcoal/dextran treated FBS in phenol red-free media to reduce variability caused by differing levels of estrogen in FBS and the ERα stimulating capacity of phenol red. Using this system, we found that BM cultures from ERαKO NZM mice, compared to WT littermates, had a lower percentage of pDCs ($CD11c^{+}B220^{+}$) and mDCs ($CD11c^{+}CD11b^{+}B220^{-}$), while maintaining a similar percentage of LDCs ($CD11c^{+}CD11b^{-}B220^{-}$) (Figure 1A and B). The same trend, although to a lesser extent, was observed in Flt3L driven BMDCs from age and sex matched B6 control mice (Figure 1C and D). This finding suggests the functional mutant of ERα reduced the total yield of cells obtained from NZM BM cultures (Figure 1E), consistent with previous results in GM-CSF driven cultures. We calculated total yield of cells by determining the ratio of the number of cells recovered on day 7 of culture compared to the number cells placed in culture on day 0. The yield of cells from cultured BMDCs was reduced in ERαKO mice and this effect was restricted to BMDCs from NZM mice. In B6 control animals, functional ERα signaling reduced the cell yield (Figure 1E). These data suggest the impact of ERα signaling on *in vitro* Flt3L driven BMDC culture development and/or survival is strain and/or disease specific. However, ERα's impact on the percentage of DC populations was consistent between NZM and B6 BMDCs. The impact of ERα signaling on DC populations was consistent with previous findings in B6 animals [11].

Overall, our findings suggest that ERα impacts the final stages of Flt3L driven DC production regardless of disease status, however, ERα differentially impacts proximal stages in Flt3L driven DC production depending on disease state.

The functional mutation of ERα increased apoptosis in Flt3L driven BM cultures from NZM mice

To investigate the reduction in cell yield in Flt3L driven BM cultures from

Figure 2

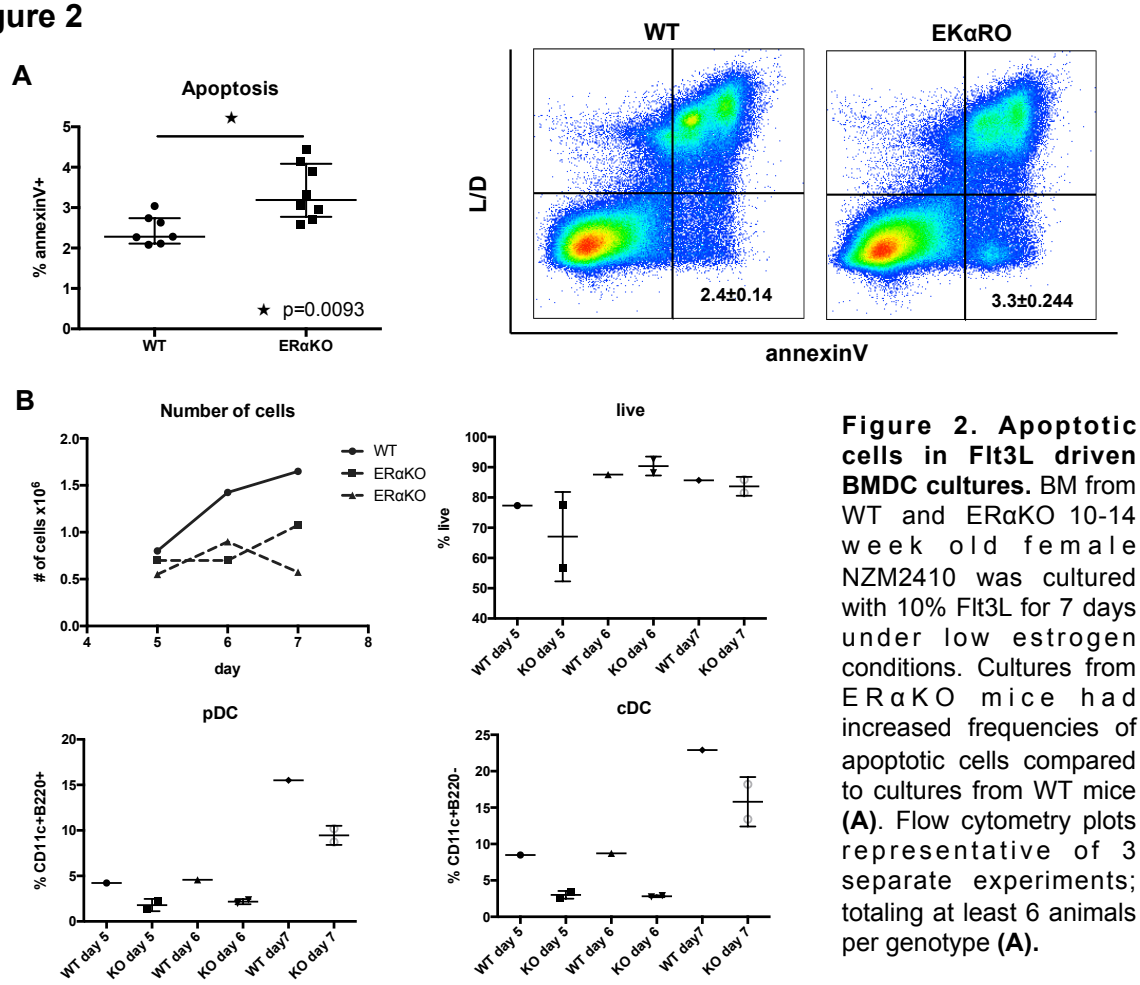


Figure 2. Apoptotic cells in Flt3L driven BMDC cultures. BM from WT and ERαKO 10-14 week old female NZM2410 was cultured with 10% Flt3L for 7 days under low estrogen conditions. Cultures from ERαKO mice had increased frequencies of apoptotic cells compared to cultures from WT mice **(A)**. Flow cytometry plots representative of 3 separate experiments; totaling at least 6 animals per genotype **(A).**

Figure 2 cont. The total number of cells continuously increased over days 5 to 7 of culture and the frequencies of live cells, pDCs, and cDCs remain constant between genotypes over days 5 to 7 **(B).** Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

ERαKO NZM mice, we measured the frequency of apoptotic and dead cells on day 7 of culture. Flt3L derived BM cultures from ERαKO NZM mice had an increased frequency of apoptotic cells (annexinV^+) compared to WT cultures (Figure 2A). This finding indicates that in Flt3L driven NZM BM cultures the functional mutation of ERα increases apoptosis, which may explain the reduced cell yield. Since the change in apoptosis, although significant, is small, we do not believe an increase in apoptosis accounts for the total reduction in cell yield. Therefore it is likely that the ERαKO cells have impaired development, since mature DCs do not proliferate. Alternatively, lupus DCs are known to be 'overactive' thus they may be reaching Flt3L induced maturation earlier than healthy DC. As a result, we may be missing their peak development in culture by harvesting at the same time as DCs from B6 mice. To determine if the lupus DCs were maturing early we harvested Flt3L driven BM cultures from WT and ERαKO NZM mice at days 5, 6, and 7 post sacrifice and measured total cell yield as well as frequency of each DC subtype. We found that the NZM Flt3L driven BM cultures do not develop early, as day 7 had the highest cells yield (Figure 2B). Additionally, the frequency of DC populations did not change between days, indicating that reduced cell yield is not due maturational changes in a specific DC subtype (Figure 2B). This finding indicates that the reduction in cell yield in NZM Flt3L driven BM cultures is not due to rapid development followed by cell death. Instead, the reduced cell yield is likely due to both alterations in DC precursor development and an increase in apoptotic cell death cause by the absence of functional ERα.

NZM mice have an altered distribution of pDCs in vivo compared to B6 controls

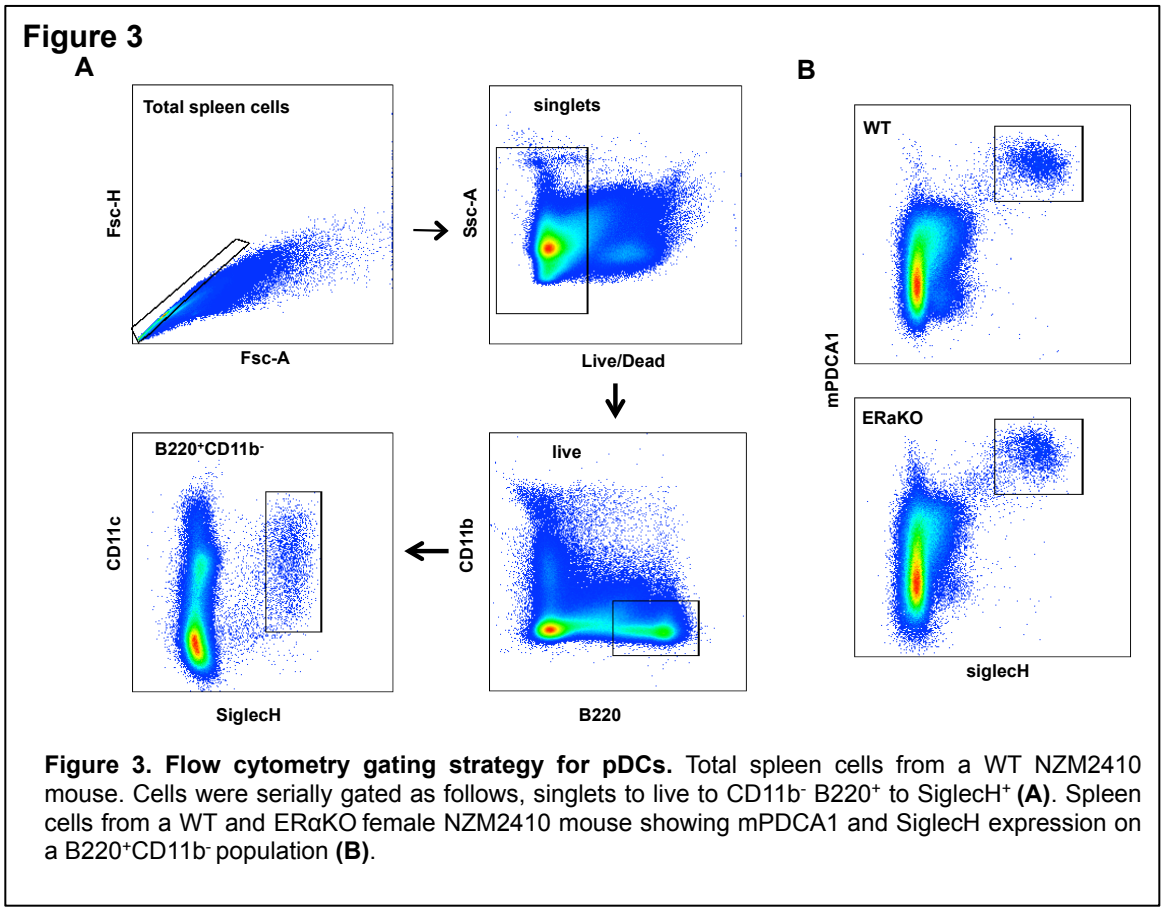


Figure 3. Flow cytometry gating strategy for pDCs. Total spleen cells from a WT NZM2410 mouse. Cells were serially gated as follows, singlets to live to CD11b$^-$ B220$^+$ to SiglecH$^+$ **(A)**. Spleen cells from a WT and ERαKO female NZM2410 mouse showing mPDCA1 and SiglecH expression on a B220$^+$CD11b$^-$ population **(B)**.

To determine if there was a difference in pDC populations between NZM and B6 mice, we measured pDCs frequency and number in the BM and spleens of pre-disease (12-14 week) female NZM mice and B6 age and sex matched control mice. pDCs were identified as (B220$^+$, CD11b$^-$, SiglecH$^+$); the gating strategy is displayed in Figure 3. We found that NZM mice had reduced frequencies and numbers of pDCs in the BM compared to B6 mice (Figure 4A). The NZM mice, however, had increased frequencies and numbers of pDCs in the spleen compared to B6 mice (Figure 4B). This finding suggests that in NZM mice the pDC population is distributed differently than B6 mice,

with more pDCs subsiding in the spleen and less remaining in the BM (their organ of origin).

Figure 4

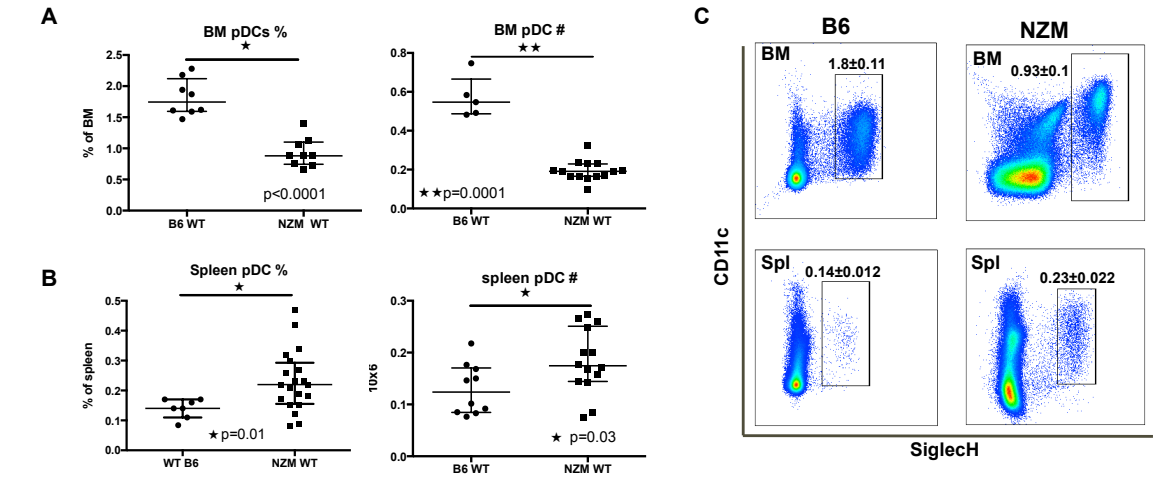


Figure 4. pDC frequency and number in the BM and spleen of B6 and NZM2410 10-14 week old female mice. Frequency and number of pDCs ($B220^+CD11b^-SiglecH^+$) in BM of B6 and NZM mice **(A)**. Frequency and number of pDCs ($B220^+CD11b^-SiglecH^+$) in the spleens of B6 and NZM mice **(B)**. Flow cytometry plots represent data from at least 4 separate experiments, which are summarized in the graphs **(C)**, B6 (n≥5) and NZM (n≥7). Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

We hypothesized pDCs from NZM mice exit the BM early and enter the periphery as immature pDCs. To test this hypothesis we measured the maturity of the spleen pDCs by measuring expression of the maturation marker Ly49Q. There was no change in the frequency of pDCs expressing the Ly49Q maturation marker in the spleens of NZM compared to B6 mice, indicating there is no difference in spleen pDC maturation status in NZM mice (Figure 5B). This finding suggests the pDCs from NZM mice are not exiting the BM as immature cells, but rather fully mature pDCs. Based on this evidence, we hypothesized NZM mice have a reduced percent of mature pDCs in the BM due to increased egress of mature pDCs from the BM. To test this hypothesis we measured Ly49Q expression on BM pDCs. NZM mice have a significantly lower frequency pDCs in the BM compared to B6 mice (Figure 5A). This finding suggest that in pre-disease

Figure 5

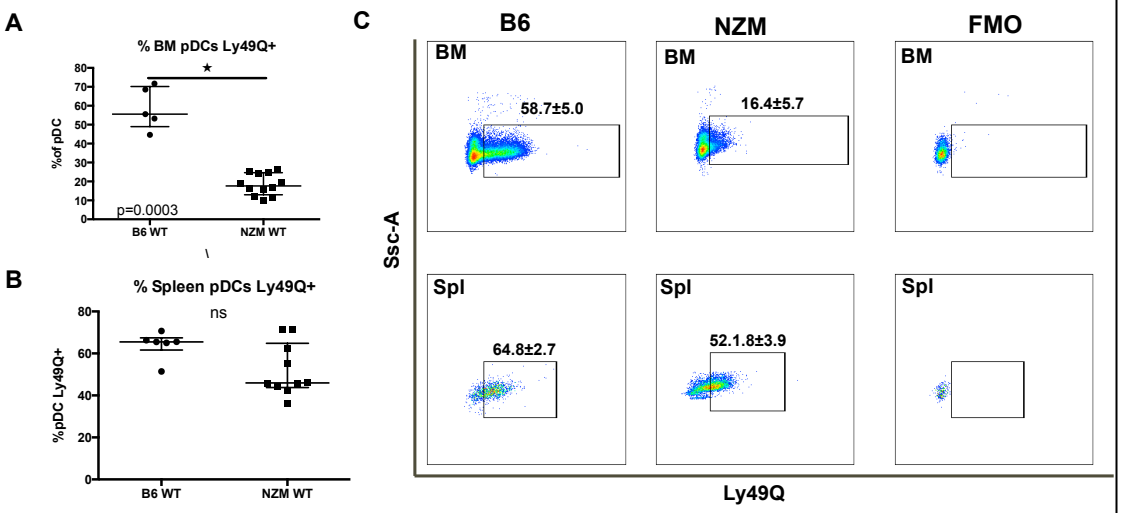


Figure 5. Frequency of Ly49Q⁺ pDCs in the BM and Spleens of B6 and NZM2410 10-14 week old female mice. Frequency of Ly49Q⁺ pDCs (B220⁺CD11b⁻SiglecH⁺) in BM of B6 and NZM mice **(A)**. Frequency and number of Ly49Q⁺ pDCs (B220⁺CD11b⁻SiglecH⁺) in the spleens of B6 and NZM mice **(B)**. Flow cytometry plots represent data from at least 4 separate experiments, which are summarized in the graphs **(C)**, B6 (n≥5) and NZM (n≥7). Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

female NZM mice the mature BM pDC population exits the BM at a higher rate than mature pDCs from B6 mice. The increased egress from the BM could explain the increase of spleen pDC number in NZM mice. Additionally, since this work was preformed in pre-disease lupus prone mice, the increase in mature peripheral pDCs may contribute to SLE pathogenesis. Future work should investigate the mechanism of this increased egress of mature pDCs from the BM. We also measured the number of cDCs in the spleens of B6 and NZM2410 10-14 week old female mice. We did not see any change in the frequency and number of cDCs in the spleens of NZM mice compared to B6 (Figure 6). However, we did see a reduction in the intensity of MHCII staining in NZM mice compared to B6 controls (to be discussed in chapter 2).

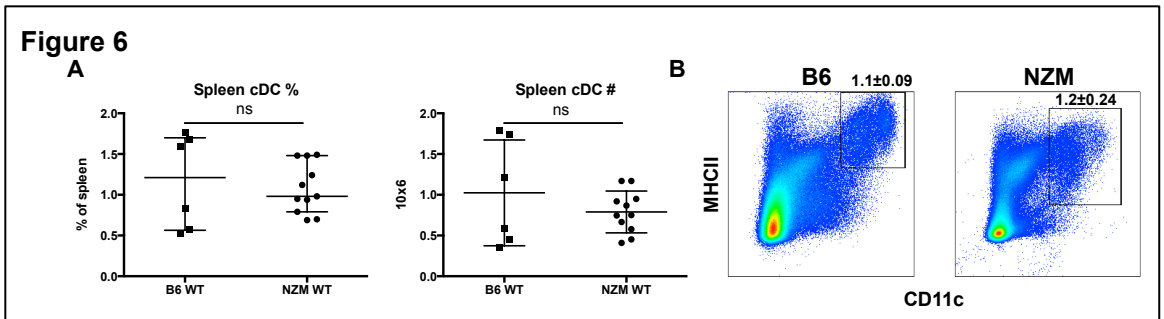


Figure 6. Frequency and number of cDCs in the spleens of B6 and NZM2410 10-14 week old female mice. Frequency and number of cDCs (B220^{-}SiglecH^{-}CD11c^{+}MHCII^{+}) in the spleens of B6 and NZM mice **(A)**. Flow cytometry plots represent data from at least 4 separate experiments, which are summarized in the graphs **(C)**, B6 (n≥6) and NZM (n≥10). Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

ERα signaling did not alter the frequency of pDCs in the spleens and bone marrow of lupus prone mice

Based on others and our *in vitro* data, we hypothesized functionally mutated ERα would reduce the percent and number of pDCs in the bone marrow and spleens of pre-disease lupus-prone mice *in vivo*. To assess the impact of ERα on DC numbers in NZM mice, we identified pDCs (CD11b^{-}B220^{+}siglecH^{+}) and the cDC population (B220^{-}, siglecH^{-}, CD11c^{+}, MHCII^{+}) in the spleens of WT and ERαKO pre-disease (10-14 week old) female mice. In contrast to the *in vitro* effects of ERα, the *in vivo* the mutation of functional ERα had no effect on the frequency or total number of spleen pDCs or cDCs in either NZM (Figure 7A and Figure 8) or B6 mice (Figure 9 and Figure 10). To confirm the accuracy of our pDC identification, as inflammation can alter the expression of the pDC markers mPDCA1 and SiglecH, we showed that the siglecH^{+} cells represented a single and overlapping population of cells with the mPDCA1^{+} population in the NZM mice (Figure 3B) [76].

Since pDCs develop to maturity in the bone marrow, we also measured the frequency and absolute number of pDCs in the bone marrow of WT and ERαKO NZM mice. Although, ERα deficiency did not alter the percent of pDCs in the BM of the NZM

Figure 7

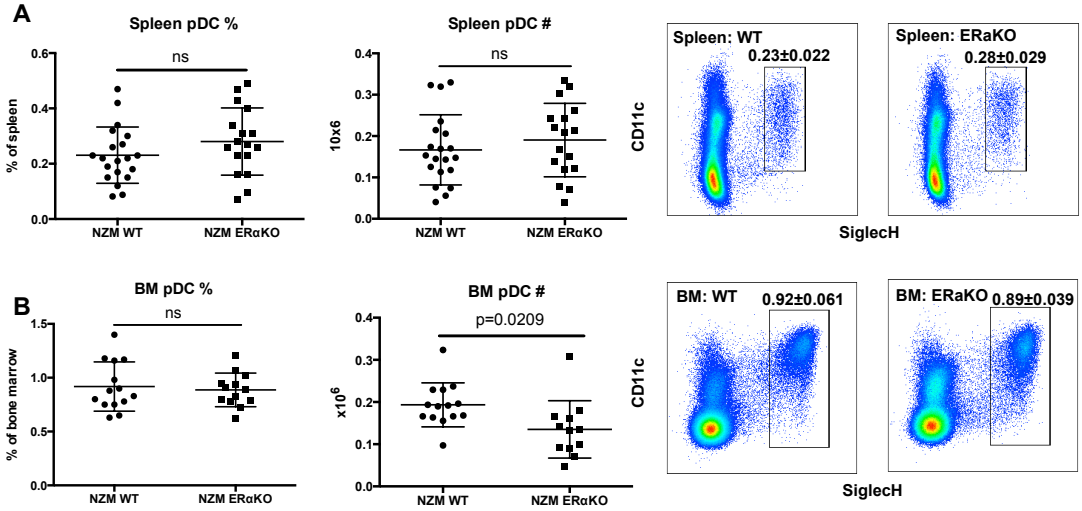


Figure 7. pDC frequency and number in the spleens and BM of WT and ERαKO 10-14 week old female NZM2410 mice. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in spleens of NZM2410 mice **(A)**. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in the bone marrow of NZM2410 mice **(B)**. Flow cytometry plots represent data from 8 separate experiments, which are summarized in the graphs, WT (n=20) and ERαKO (n=17). Results are expressed as the mean ±SD. Statistical analysis performed with students T test.

Figure 8

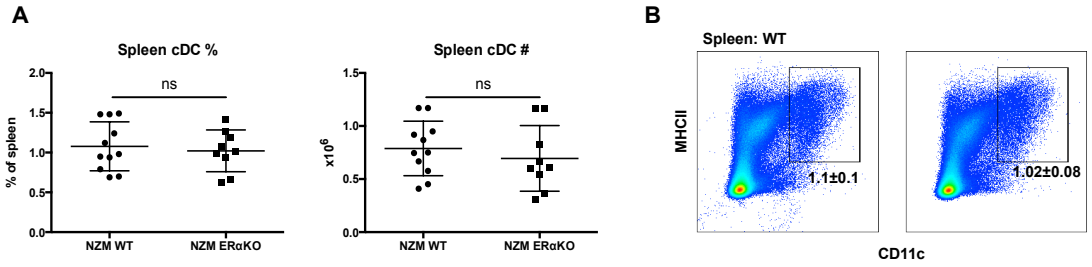


Figure 8. Frequency and number of cDCs in the spleens WT and ERαKO 10-14 week old female NZM2410 mice. Frequency and number of cDCs ($B220^{-}SiglecH^{-}CD11c^{hi}MHCII^{hi}$) in spleens of NZM2410 mice **(A)**. Flow cytometry plots represent data from 5 separate experiments **(B)** which are summarized in the graphs, WT (n≥10) and ERαKO (n≥9). Results are expressed as the median ±SD. Statistical analysis performed with students T test.

mice, ERα deficiency reduced the absolute number of BM pDCs (Figure 7B). The reduction in pDC absolute number was due to a decrease in the overall bone marrow cellular yield in ERαKO mice (Figure 7B). Despite the consistent decrease in BM

Figure 9

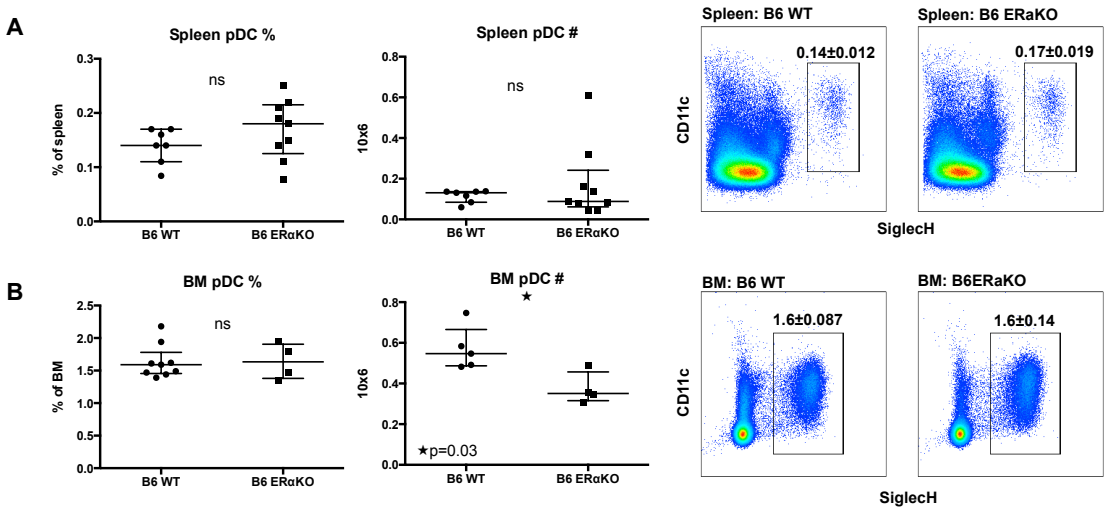


Figure 9. pDC frequency and number in the spleens and BM of WT and ERαKO 10-14 week old female B6 mice. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in spleens of B6 mice **(A)**. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in the bone marrow of B6 mice **(B)**. Flow cytometry plots represent data from 4 separate experiments, which are summarized in the graphs, WT (n=7) and ERαKO (n≥5). Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

cellularity and pDC numbers in ERαKO NZM mice, total spleen cell counts and spleen pDC and cDC numbers were unaltered. This finding suggests the hematopoietic

Figure 10

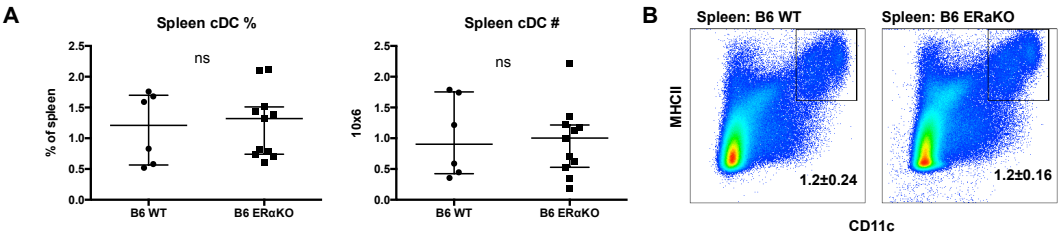


Figure 10. Frequency and number of cDCs in the spleens WT and ERαKO 10-14 week old female B6 mice. Frequency and number of cDCs ($B220^{-}SiglecH^{-}CD11c^{hi}MHCII^{hi}$) in spleens of B6 mice **(A)**. Flow cytometry plots represent data from 4 separate experiments **(B)** which are summarized in the graphs, WT (n≥6) and ERαKO (n≥9). Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis.

compartment can compensate for the decreased bone marrow cellularity, perhaps by accelerated egress from the marrow or increased cellular life span of ERα mutant cells.

To support the hypothesis that accelerated egress of pDCs from the BM contributes to the equal spleen pDC numbers there was a reduction in mature Ly49Q+ pDCs in the BM of ERαKO NZM mice (Figure 11). Alternatively, overall pDCs in the periphery of ERαKO NZM mice may be decreased, but the pDCs remain in the spleen rather than migrating to peripheral organs impacted by disease in ERαKO mice.

Figure 11

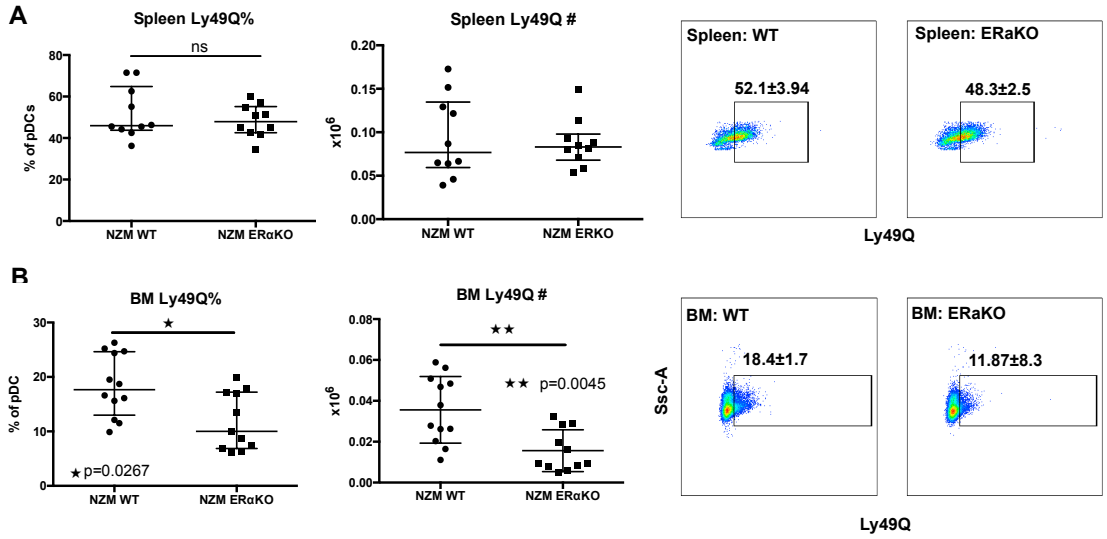


Figure 11. Frequency and number of Ly49Q+ pDCs in the spleens and BM of WT and ERαKO 10-14 week old female NZM2410 mice. Frequency and number ofLy49Q+ pDCs ($B220^+CD11b^-SiglecH^+$) in spleens of NZM2410 mice **(A)**. Frequency and number of Ly49Q+ pDCs ($B220^+CD11b^-SiglecH^+$) in the bone marrow of NZM2410 mice **(B)**. Flow cytometry plots represent data from 8 separate experiments, which are summarized in the graphs, WT (n≥10) and ERαKO (n≥10). Results are expressed as the median ±IQR. Statistical analysis preformed with Mann-Whitney analysis.

ERα signaling did not alter the frequency of pDCs in the spleens of diseased NZM mice

To determine if advanced age of the NZM mice may bring out a difference in pDC frequencies and numbers between WT and ERαKO mice, we measured spleen pDC

populations in 22-26 week old, as the mice begin to develop proteinuria, WT and ERαKO NZM mice. We also measured the frequency of non-pDC CD11c+ cells. Similar to the pre-disease mice there was no change in the frequency or number of pDCs or CD11c+ cells in the ERαKO mice compared to WT mice (Figure 12). This finding suggests the effect of the mutant ERα does not impact disease by altering spleen pDC frequency or number.

Figure 12

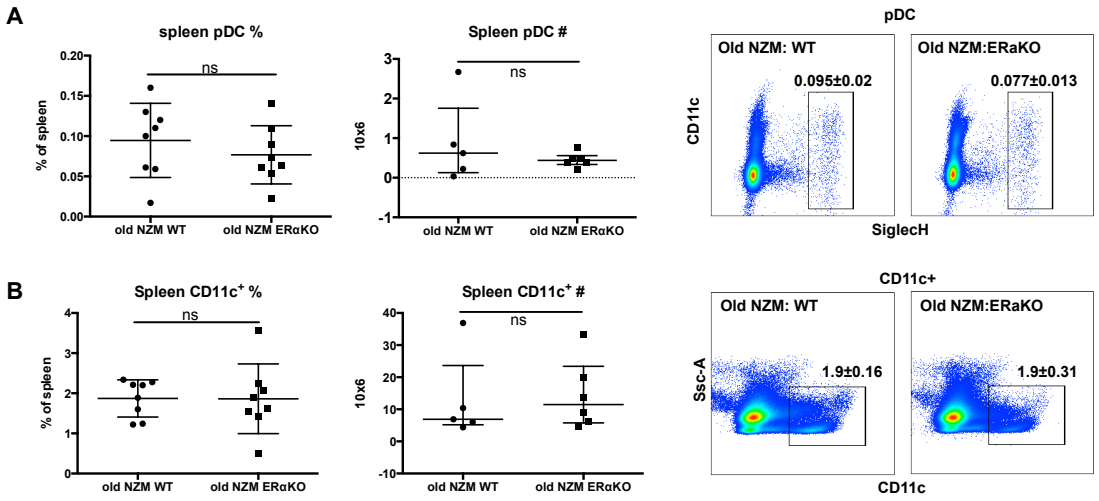


Figure 12. Frequency and number of pDCs and CD11c+ cells in the in the spleens WT and ERαKO 22-26 week old female NZM2410. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in spleens old NZM2410 mice (22-26 wks) **(A)**. Frequency and number of non pDC CD11c+ cells ($B220^{-}SiglecH^{-}CD11c^{+}$) in spleens old NZM2410 mice (22-26 wks) **(B)** Flow cytometry plots represent data from 4 separate experiments **(B)** which are summarized in the graphs, WT (n≥6) and ERαKO (n≥8). For non normal data (pDC and cDC #), Results are expressed as the median ±IQR. Statistical analysis performed with Mann-Whitney analysis. For normal data (pDC and cDC %) Results are expressed as the mean ±SD and statistical analysis was performed with a Student's T test analysis.

Interestingly, there was a change in frequency and number of pDCs and CD11c+ cells in the spleens of the old NZM mice compared to the pre-disease NZM mice (Figure 13) Old NZM mice have reduced pDC frequency compared to young NZM mice (Figure 13). Additionally, the old NZM mice have an increase in frequency of CD11c+ cells compared to young NZM mice (Figure 13). Regarding absolute numbers, the spleens of

old NZM mice are enlarged, therefore there is an increase in total spleen cell count. Thus, despite the decrease in pDC frequency there is a trend toward an increase in total number of pDCs in the spleens of old NZM mice. There was significantly increased number of CD11c+ cells in the spleens of old NZM mice compared to young NZM mice. This finding suggests that DC numbers/distribution play a role in lupus pathogenesis.

Figure 13

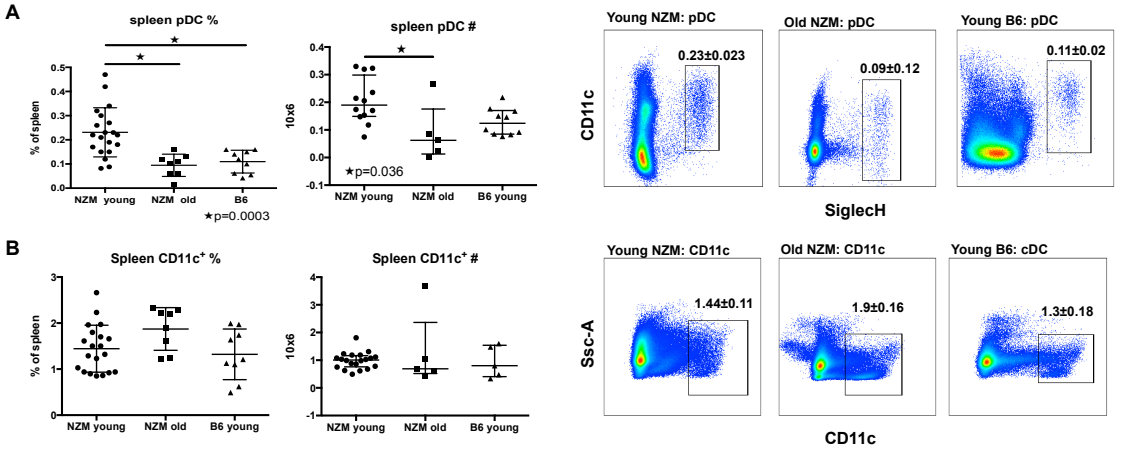


Figure 13. Frequency and number of pDCs and CD11c+ cells in the in the spleens of young and old female NZM2410. Frequency and number of pDCs ($B220^{+}CD11b^{-}SiglecH^{+}$) in spleens of young (10-14 weeks) and old (22-26 weeks) female NZM2410 mice **(A)**. Frequency and number of non pDC CD11c+ cells ($B220^{-}SiglecH^{-}CD11c^{+}$) in spleens of young (10-14 weeks) and old (22-26 weeks) NZM2410 mice **(B)** Flow cytometry plots represent data from 4 separate experiments **(B)** which are summarized in the graphs, young (n≥6) and old (n≥5). Results are expressed as the mean ±SD for % and median ±IQR for #. Statistical analysis preformed with Kruskal-Wallis test.

Summary

Flt3L BMDC culture:

• BM from B6 and NZM ERαKO mice generate a reduced percent of pDCs and mDCs

• BM from B6 ERαKO mice had increased cell yield compared to B6 WT mice

• BM from NZM ERαKO mice had reduced cell yield compared to WT NZM mice.

• BM cultures from NZM ERαKO mice had a greater frequency of apoptotic cells compared to WT NZM mice

Ex vivo pDC population

B6 versus NZM:

• NZM mice have increased numbers of spleen pDCs and reduced number of BM pDCs compared to B6 mice. This is likely due to increased pDC egress from the BM in NZM mice

Ex vivo pDC population; NZM WT versus NZM ERαKO:

• NZM ERαKO mice have equal number and frequency of spleen pDCs and cDCs compared to WT NZM mice both prior to the development of clinical disease and after the development of disease.

• NZM ERαKO mice have equal frequency, but reduced absolute number of pDCs in the BM of NZM mice. The reduction in BM pDCs number is due to a decrease in overall BM number.

2.4 Discussion

ERα signaling in Flt3L driven BMDC cultures from NZM mice

ERα signaling impacts Flt3L driven BMDC cultures via two mechanisms. In BM from B6 mice, ERα signaling negatively impacts numbers of BM progenitor cells to reduce the total number of cells generated via this culture system [11, 51]. In the same cultures, ERα signaling positively impacts the maturation of late DC progenitors to increase the frequency of cDC and pDCs acquired from the cultures [51]. Therefore Flt3L driven cultures of BM from ERαKO mice yields an increased number of cells, but a reduced frequency of pDCs and cDCs compared to BM from ERα WT mice [11, 12]. Since DCs contribute to lupus pathogenesis, we wanted to determine if ERα signaling also impacted Flt3L driven DC development in BM from NZM lupus prone mice. Regarding percent of pDC and cDCs produced by cultures, we found BM from NZM ERαKO mice yielded reduced frequencies of pDCs and cDCs, similar to results reported in B6 mice and our own findings in B6 mice. This result suggests that, in lupus DCs, ERα signaling positively regulates the late stage of DC development. Regarding total cell yield, in our Flt3L of cultures of B6 BM, we found ERα signaling negatively regulated total cell yield, similar to previous studies [11]. However, in Flt3L cultures from NZM mice, ERα signaling positively impacted cell yield, indicating ERα signaling functions differently in DC development when cells are obtained from lupus prone animals. We hypothesize three possible reasons for this difference in ERα signaling between B6 and NZM mice; BM from lupus prone mice may have differences in the number and ratios of progenitor cell populations compared to B6 mice, BM cells from lupus prone mice may create a different cell culture environment than BM from B6 mice, or BM progenitors

from lupus prone mice have intrinsic genetic differences that impact ERα signaling. Regarding the number and ratios of progenitor cell populations, it is possible the early progenitor cells, which are negatively impacted by ERα signaling, may be reduced in lupus BM. Therefore, ERα signaling in these cultures will not negatively impact cell number because the ERα sensitive population of progenitors represents a smaller proportion of the BM population. BM cell progenitor populations have not been studied in lupus prone mice, therefore, it will be important to study these cell populations in the future. Regarding the culture environment produced by lupus BM, it is possible BM cultures from lupus prone mice produce an inflammatory environment due to the hyperactivity of lupus immune cells. Additionally, the BM cells, prior to being removed for culture exist in an inflammatory environment in the mouse. This environment could have a pre-programming effect on the BM cells, which impacts ERα signaling in response to Flt3L. The possibility of an inflammatory environment is important because ERα functions differently in DCs depending on the culture conditions [56]. ERα contains two activation functions, AF-1 and AF-2. The requirement for each of these domains various between inflammatory and steady state DC. Although AF-1 is required for steady state cDC development and function and early iDC development it is dispensable in mature iDCs [56]. Based on this evidence, ERα signaling may change in the inflammatory environment created in the BM of lupus prone mice. Another possibility for the difference in cell yield is that BM cells and DCs from lupus prone mice have altered genetics, which change the way ERα signaling impacts cell development. To conclude, although ERα signaling functions similarly in DCs from B6 and NZM in some situations (later stages of DC development) ERα signaling can also have different

functions (early stages of DC development). Therefore we must study the role of ERα in both the healthy and disease state in order to understand ERα's role in immune function during disease.

The effect of ERα signaling on pDC populations in vivo in lupus prone mice

Since ERα signaling positively impacts DC generation from BM cultures from lupus prone mice, we hypothesized NZM ERαKO mice have fewer spleen pDCs compared to WT NZM mice. However, we did not detect a change in pDC or cDC frequency or number in the spleens of ERαKO mice compared to WT mice. This finding suggests ERα signaling does not impact *in vivo* pDC generation in the same manner as occurs *in vitro*. One possibility for the lack of change is that ERα signaling is not required for *in vivo* pDC generation. However, Flt3L and GM-CSF are known to be critical for *in vivo* DC generation and ERα signaling impacts these pathways of DC development, making this scenario unlikely [51]. Another possibility is that other growth factors, present *in vivo,* compensate for the lack of ERα signaling. This possibility is likely because ERαKO mice have altered levels of estrogen, testosterone, and prolactin [1]. Changes in levels of the hormones may compensate for lack of ERα signaling therefore, this possibility should be further investigated. This could be done by controlling for hormones levels in ERαKO by ovariectomizing both WT and ERαKO mice and adding back physiologic levels of estradiol. Alternatively, the lack of change we reported may be due to an inability to detect the change caused by ERα signaling. Since we are not measuring total body pDCs, our findings in the spleen may be confounded by changes in pDC migration and distribution. Therefore, ERα's impact may be better detected in the

tissue. Both GM-CSF and Flt3L are produced locally during inflammation and immune responses. Therefor ERα deficiency may impact cDC or pDC generation in the tissue. Although this explanation is likely for cDCs, as they are known to mature in the periphery, this is unlikely for pDCs, as they reach full maturity in the BM.

Since pDCs develop to maturity in the BM, we believe measuring pDCs in the BM is a better measure of the ability of the ERαKO mice to generate pDCs. In the BM, we also did not detect a change in pDC frequency in the ERαKO mice. Similar to possibilities in the spleen, ERα's role in pDC generation may be unnecessary or compensated *in vivo*. Alternatively, the change in pDC frequency may be small or brief, therefore making it undetectable by our methods. Like in the spleen, changes in migration may confound our results. We hypothesized if pDCs were exiting the BM more quickly in WT mice, thereby negating any change in pDC frequency cause by ERα signaling, there would be less Ly49Q+ (mature) pDCs present in the BM of WT NZM mice compared to ERαKO mice. When we measured mature pDCs in the bone marrow, we did not see a reduction in mature pDCs in the BM of WT NZM mice. Instead, there were slightly less mature pDCs in the BM of the ERαKO mice. Based on our findings, we conclude ERα signaling does impact Flt3L driven DC generation *in vitro*, however, this effect is not detectable *in vivo*. Therefore, it is unlikely that ERα signaling impacts disease by suppressing generation of mature pDCs.

The ERαKO mice have a reduced number of total BM cells. One explanation for the reduction in total BM cellularity is that ERα signaling is required in BM hematopoietic progenitors for Ftl3L driven expansion. It was previously reported that ERα deficiency impaired the renewal of hematopoietic stem-cells in the bone marrow

[77]. Impairment in the expansion and renewal of early progenitors would explain a reduction in total BM cell count. Alternatively, ERα signaling may impact the stromal cell compartment. Seillet et al. showed when ERα deficiency was restricted to hematopoietic cells, BM cellularity was not impacted [16]. This finding suggests the reduction in BM cellularity is due to changes in bone mass (reduced space for BM), or changes in stromal cell support of BM development. Independent of the reasoning for the reduction in BM cellularity, the reduced number of BM cells may contribute to disease protection in NZM ERαKO mice.

pDC numbers in NZM2410 mice compared to B6 mice

Two studies reported the deletion of pDCs protects lupus prone mice from disease, thus, confirming their importance in disease pathogenesis [9, 10]. However, whether they contribute to disease through abnormal numbers or function or both is not understood. Therefore, in order to interpret our findings in the NZM ERαKO mice we needed to first understand how pDC numbers are altered in NZM mice. Pre-disease female NZM mice have an increased frequency and number of pDCs in the spleen compared to age and sex matched B6 mice, indicating, changes in pDC number may contribute to disease. This is the first report, to our knowledge, of an increase in pDCs in the spleens of pre-disease lupus prone mice. In a 2013 publication, pDCs numbers were increased in diseased NBW F1 mice, but not in the pre-clinical disease animals [69]. The studies differed in stain of lupus prone mouse; however, the models are similar as mating the NBW F1 generation created the NZM2410 mice. The major difference between the studies was the method by which pDCs were identified. In Gleisner et al. CD11c and

B220 were used as pDC markers [69]. Using these markers there is significant contamination with T, B and NK cells. In our study we used CD11c B220 and the pDC specific marker SiglecH, which is a more accurate method of identifying pDCs [78]. Therefore, there is likely a true increase in pDC frequency and numbers in the spleens of pre-disease NZM2410 mice compared to B6 controls.

We also measured spleen pDC frequency and number in 22-26 week old NZM mice. In this old cohort the spleen pDC frequency and numbers declined to levels comparable to B6 mice. This finding also differs from Gleisner et al, where an increase in pDC frequency and number, as measured by B220, was reported in diseased mice. The pDC markers used could also explain the discrepancy between our findings. The marker we used, SiglecH, is known to decrease on pDCs *in vitro* when they are stimulated with TLR ligands (e.g. Immune complexes) [78]. Therefore, it is possible that pDCs in lupus animals are no longer expressing siglecH. However, the use of B220 as a sole pDC marker is not specific. Therefore, new models should be used to study pDCs in lupus prone mice. The siglecH-eGFP mouse, which expresses GFP on pDCs even if siglecH is down regulated, would be a good model to use in lupus [79].

If spleen pDC numbers are truly reduced in aged NZM mice this may be explained by pDCs leaving the spleen late in the course of the disease and traveling to sites of inflammation, which is known to occur in SLE. Alternatively, the spleen pDCs could convert to cDCs during the course of disease. *In vitro* after TLR stimulation, pDCs can convert to a cDC phenotype [80]. However, this conversion has not been demonstrated to occur *in vivo*. Interestingly, in this study and other publications, cDCs are increased in diseased mice, supporting the hypothesis DC conversion may occur.

Further investigation is needed to understand the fate of pDCs during the progression of lupus.

The increase in spleen pDCs in pre-diseaase NZM mice likely occurs because of increased migration from the BM. In our work, there was a specific reduction in mature pDCs in the BM of the NZM mice, suggesting NZM mice are not generating more pDCs than healthy mice, but rather the migration to the periphery increases in NZM mice. Work by Gleisner et al, supports this hypothesis, by demonstrating increased homing of pDCs to the spleens in lupus prone mice [69]. Based on this finding, future work should investigate expression of chemokine receptors on pDCs in SLE and the production of these chemokines in the periphery.

We conclude that the increased numbers of pDCs in the spleens of NZM mice likely contribute to disease pathogenesis. Furthermore, the increase is likely due to increased migration of the pDCs to the spleen. However, the NZM WT and ERαKO mice have equal numbers of pDCs in the spleens, indicating this is not a mechanism by which ERα impacts disease.

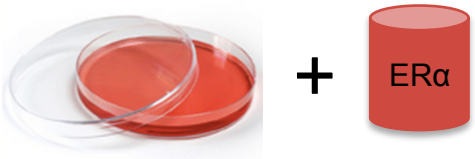
2.5 Conclusions

pDC distribution is altered in NZM mice compared to B6 mice. Furthermore, the frequency and number of spleen pDCs is increased in the spleen prior to the development of clinical disease. Based on this evidence and previous work in pDC deficient lupus prone mice, we conclude pDC frequency and number likely impact progression of lupus in NZM mice. In Flt3L driven BMDC cultures, the loss of ERα signaling negatively impacted the generation of mature pDC and cDC populations. Furthermore, ERα signaling differentially impacted Flt3L driven DC cultures from NZM mice compared to B6 controls. These findings suggest ERα signaling is important for DC generation, but its precise role varies depending on disease state. Despite the impact of ERα on *in vitro* DC generation, NZM ERαKO had no change in spleen pDC frequencies, numbers, and maturation state compared to WT NZM mice, suggesting *in vivo* DC generation compensates for a lack of functional ERα signaling. Interestingly, the total BM cell count was reduced in ERαKO mice compared to WT mice. Since this decrease was not specific to the pDC lineage, this finding suggests functional ERα signaling may be required for hematopoietic progenitor cell survival. Since the progenitor cells are responsive to Flt3L, this finding may represent an *in vivo* role of ERα in Flt3L signaling. Additionally, the impact of ERα signaling on total BM cell count may impact lupus pathogenesis.

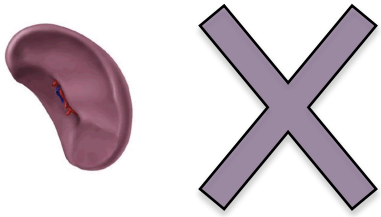
Diagram 7

Aim I Summary

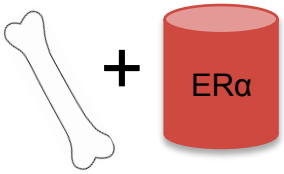
ERα signaling increased pDC frequency in Flt3L driven BMDC cultures



ERα signaling did not impact spleen pDC frequency or number



ERα signaling increased BM cell counts in pre-disease NZM mice



2.6 Future Directions

The findings presented in this chapter suggest the NZM ERαKO mice are not protected from disease by having impaired generation of pDCs. However, we have shown that pDC number is altered in pre-disease mice, suggesting pDC distribution and number impact pathogenesis. Additionally, we have shown *in vitro* ERα signaling is important for pDC generation. The reason why ERα impacts FLt3L driven *in vitro* culture of DCs but does not impact *in vivo* generation of these cells remains unanswered. The difference could be due to changes in hormone levels in ERαKO mice. The ERαKO mice have increased levels or estrogen and testosterone compared to WT mice. Additionally, in the ERαKO model small amounts of mutant ERα protein lacking only the AF1 domain are produced. Since AF1 function is dispensable for different stages of DC development, the high levels of estradiol in the ERαKO mice may drive the function of the mutant ERα thereby impacting *in vivo* pDC development and explain the discrepancy (since estradiol levels are low in the culture conditions).

Although we did not see a difference in pDC populations specifically, we did see a reduction in total BM cellularity in the ERαKO mice. To determine if this is a change caused by ERα in the hematopoietic or stromal cell compartment we should study ERαKO in a DC specific model. Although a BM transplant could be used to study this effect in B6 mice, the NZM2410 mice a radiation resistant. Additionally, the use of AF1 and AF2 ERα mutants would also be helpful in understanding ERα's role in disease.

Regarding the role of pDCs in lupus, independent of ERα, much work is needed to understand the function of these cells during disease. Three areas of potential interest

have been identified in this study. First, the migration pattern of pDCs is not understood in lupus. Second, BM composition has not been investigated in lupus prone mice, and our work suggests changes occur in this compartment prior to clinical disease. Third, the fate of pDCs throughout the course of disease should be studied. Specifically, the idea of pDC to cDC conversion needs to be studied *in vivo*.

Chapter 3: Impact of estrogen receptor alpha on plasmacytoid dendritic cell activation

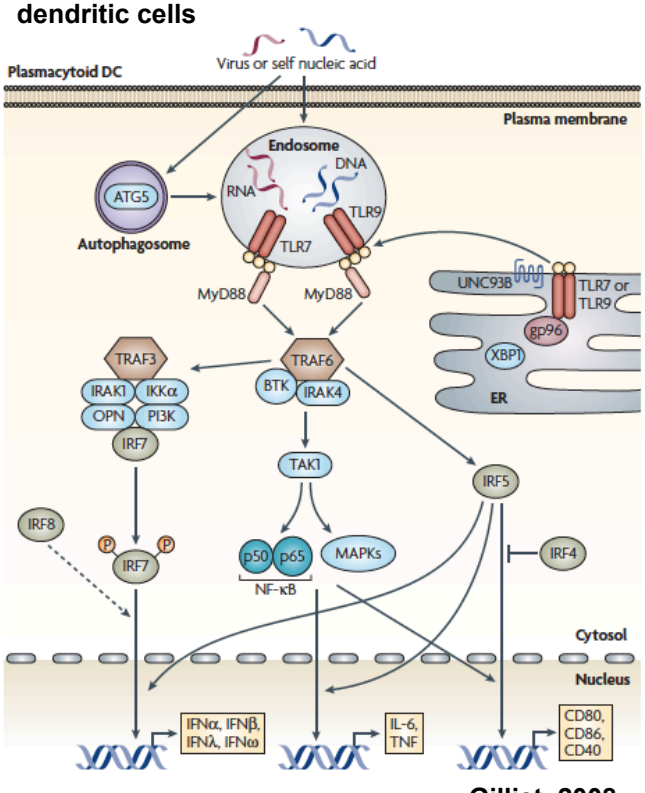
Chapter 3 Impact of Estrogen Receptor α on plasmacytoid dendritic cell activation

3.1 Introduction

pDC activation and function

pDCs share a precursor and name with cDCs, but they function differently than cDCs. In mice, pDCs express only the endosomal TLRs 7 and 9 [38]. Once either TLR is stimulated, typically in response to viral infection, pDC produce large amounts of type I IFNs. The type I IFN produced by pDCs function to activate T, B, NK cells, and other DCs. Alternatively, pDCs can also express high levels of MHCII, costimulatory molecules, and produce TNF and Il-16 in response to TLR stimulation [37]. Expression of MHCII, costimulatory molecules and production of TNF and Il-6 allow pDCs to act to acquire T stimulatory capacity [81].

Diagram 8 TLR signaling in plasmacytoid dendritic cells



TLR 7 and 9 ligands induce IFNα, IL-6, and TNF production and expression of costimulatory molecules in pDCs

TLR7 and TLR9 sense ssRNA and unmethylated CpG containing DNA, respectively. TLR9 can be stimulated by different types of CpG, included CpG type A

and type B. Type A is a multimeric structure, which preferentially induces IFN production and CpG type B is a monomeric structure, which induces T cell stimulatory capacity [44]. Once stimulated both TLR7 and 9 recruit the adaptor molecule MyD88. If CpG A is the stimulus MyD88 recruitment results in a signaling cascade leading to the phosphorylation and nuclear transport of IRF7 and type I IFN transcription (Scheme 5) [44]. If CpG B stimulates TLR9 MyD88 will recruit a different set of signaling molecules, which results in NFkB activation, which promotes T cell stimulatory capacity (Scheme5) [44].

Regulators of pDC type I IFN production are known. These include IRF7, PI3K, Ly49Q, and PDC-TREM. The constitutive expression of IRF7 contributes to the pDC's ability to quickly produce large amounts of IFNs. PI3K activation of mTOR positively regulates IFN production by promoting the nuclear localization of IRF7 [82]. Two markers of pDC activation, specific to the IFN pathway are Ly49Q and PDC-TREM. Ly49Q expression is acquired by pDCs as a late stage maturation marker in the bone marrow and is required for IFN production [73, 74, 83]. PDC-TREM expression is acquired after TLR stimulation and is also required for IFN production [84]. PDC-TREM is associated with the cellular signaling molecule DAP12, which functions as an activating moiety when associated with PDC-TREM, that phosphorylates PI3K and ERK1/2 upon stimulation of pDC-TREM [84]. Stimulation of SiglecH and BST2 down regulate the IFN response [37]. Interestingly, SiglecH also signals through DAP12, however, in this case DAP12 acts as an inhibitory molecule [78].

Regarding antigen presentation, pDCs can express high levels of MHCII and present antigen to stimulate T cells after TLR engagement. pDC antigen presentation

differs from cDC antigen presentation because pDCs require TLR stimulation to present antigen. Additionally, pDCs continuously synthesize MHCII so that they are able to constantly sample the environment and present new antigen [85]. There is *in vitro* evidence that pDCs, once stimulated by TLR ligands, convert completely to a cDC phenotype [37]. However, there is no evidence to support this occurs *in vivo* or in lupus [37].

DC activation and lupus

In SLE, there are increased levels of type I IFNs and inflammatory cytokines. The increase in IFN levels is due to an inappropriate stimulation of pDC TLRs by immune complexes [44]. Immune complexes from SLE patients induced type I IFN production from pDCs. Additionally, when pDCs are deleted from lupus prone mice, IFN activity is reduced and mice are protected from disease[10]. However, the specifics of pDC activity during human and murine disease are not well understood. Lupus pDCs display an altered activation status. In mice, lupus mDCs and pDCs express increased levels of CD40 and reduced CD54 and CD80 compared to normal controls [70]. Furthermore, mDCs express high levels of CD40 prior to the development of clinical disease [70]. Human monocyte-derived DCs from SLE patients express higher levels of HLA-DR and the co-stimulatory molecules CD86 and CD80 [86, 87]. As a result, SLE mDCs also induce T cell hyperactivity [88]. pDCs from SLE patients were found to have normal levels of costimulatory molecules [41]. When SLE pDCs were stimulated *ex vivo* with CpG, pDCs from SLE patients produced less type I IFN compared to controls [89]. The reduced IFN

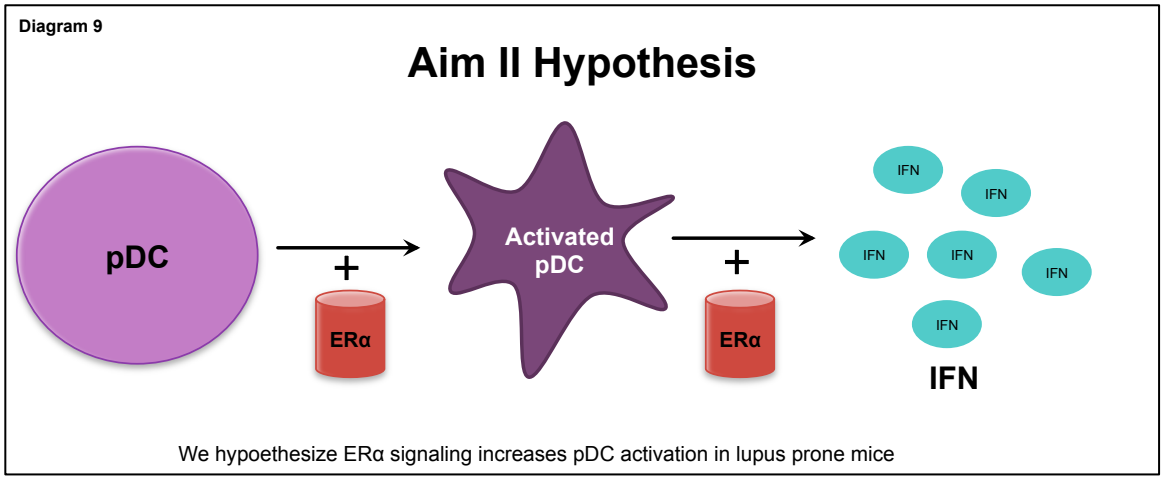
production was attributed to over stimulation with TLR ligands in SLE. Thus, more work should be done to understand the activity levels of mDCs and pDCs in SLE.

Dendritic cells and ERα

ERα signaling also impacts DC activation and function. ERα signaling promotes the function of pDCs and mDCs (see introduction). Regarding pDC IFN production, ERα signaling increased pDC production of IFNα in response to TLR 9 stimulation [16]. The increase in type I IFN production occurred when estradiol was given *in vivo* to humans and mice and when it was given at the time of *ex vivo* TLR stimulation [16, 53]. When mice were given estradiol *in vivo* prior to *ex vivo* TLR stimulation, the increase in IFNα production was dependent on ERα signaling [16]. In these studies only the in vitro stimulation of pDC activity was studied, therefore work should be done to address ERα's role in pDC activation *in vivo*. Additionally, ERα's impact on DC activity in SLE should be investigated.

3.2 Hypothesis

pDC activity impacts lupus pathogenesis. Additionally, ERα signaling positively impacts pDC activity. However, the connection between pDC activation, ERα, and lupus pathogenesis has not been explored. **We hypothesized ERα signaling positively regulates pDC activation in pre-disease NZM2410 lupus prone mice**. ERα's role in pDC activation in lupus prone mice may explain the disease protection of the ERαKO mice.



3.3 Results

Absence of functional ERα reduced the frequency of activated spleen pDCs in NZM2410 mice.

Since the absence of functional ERα did not alter spleen pDC percent or number, but does have a significant effect on disease expression, we hypothesized the lack of functional ERα impairs the activation of pDCs in NZM mice. To determine the effect of ERα signaling on the activation state of DCs in NZM mice, we measured the frequency

Figure 14

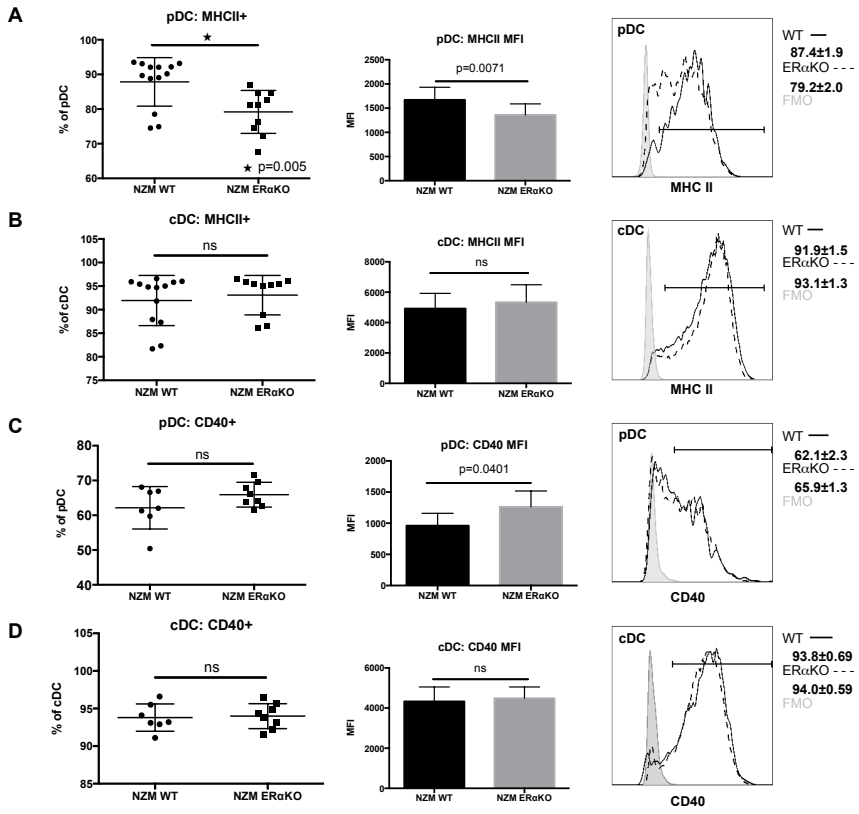


Figure 14. Frequency of activated spleen pDCs and cDCs. Frequency of MHCII$^+$ **(A)** and CD40$^+$ **(C)** pDCs and median fluorescent intensity (MFI) of pDC MHCII **(A)** and CD40 **(C)** in the spleens of WT (n=13) and ERαKO (n=10) 10-14 week old female NZM2410 mice in 5 separate experiments. Frequency of MHCII$^+$ **(B)** and CD40$^+$ **(D)** cDCs and MFI of cDC MHCII **(B)** and CD40 **(D)** in the spleens of WT (n=7) and ERαKO (n=8) 10-14 week old female NZM2410 mice in 3 separate experiments. Gates for frequency were set in each experiment based on a fluorescence minus one (FMO) control. Results are expressed as the mean ±SD or median± IQR . Statistical analysis performed with student's T test or Mann-Whitney analysis. Based on normality assessment.

of pDCs and cDCs expressing major histocompatibility complex class II (MHCII) and CD40, two described markers of DC activation. MHCII expression is required for antigen presentation and CD40 expression is necessary for T cell activation [85, 90]. Additionally, in *Sle*1,2,3 mice, cDCs have increased CD40 expression prior to disease

[70]. As shown in Figures 14A and C, the absence of functional ERα reduced the frequency of pDCs expressing MHCII, but not CD40. ERα genotype did not alter the frequency of cDCs expressing MHCII or CD40 (Figure 14B and 14D). The reduced frequency of MHCII^{+} pDCs was restricted to the NZM mice, as MHCII expression on pDCs from B6 mice was not altered by the functional deficiency of ERα (Figure 15). These findings suggest ERα specifically impacts pDC activation during disease.

Figure 15

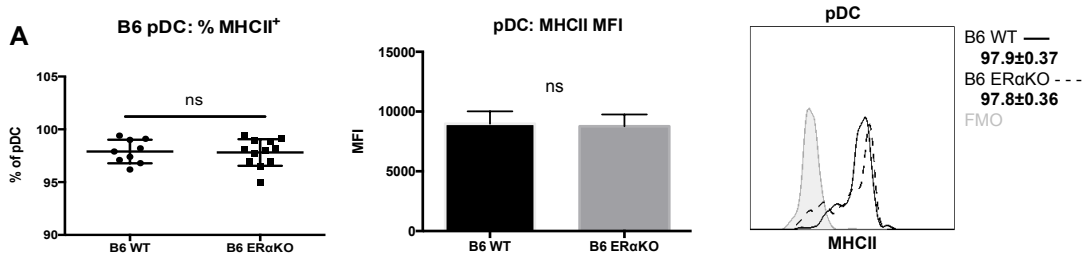


Figure 15. Frequency of activated spleen pDCs in B6 mice. Frequency of MHCII^{+} pDCs **(A)** in the spleens of WT (n=9) and ERαKO (n=12) 10-14 week old female B6 mice in 4 separate experiments. Gates for frequency were set in each experiment based on a fluorescence minus one (FMO) control. Results are expressed as the mean ±SD. Statistical analysis performed with Mann-Whitney analysis.

NZM mice have reduced DC MHCII expression compared to B6 control mice

Since ERα altered the frequency of MHCII^{+} pDC in NZM mice, we compared MHCII expression in NZM mice to B6 mice to understand how disease impacts DC MHC II expression. Independent of ERα, there was a reduced frequency of MHCII+ pDCs in NZM mice compared to B6 controls (Figure 15). We also detected an increased frequency of MHCII+ cDCs in NZM mice compared to B6 controls (Figure 16). However, there was a reduced intensity of the MHCII staining in NZM mice compared to B6 mice (Figure 16). Thus, there is a greater frequency of MCHII + cDCs in NZM mice.

Figure 16

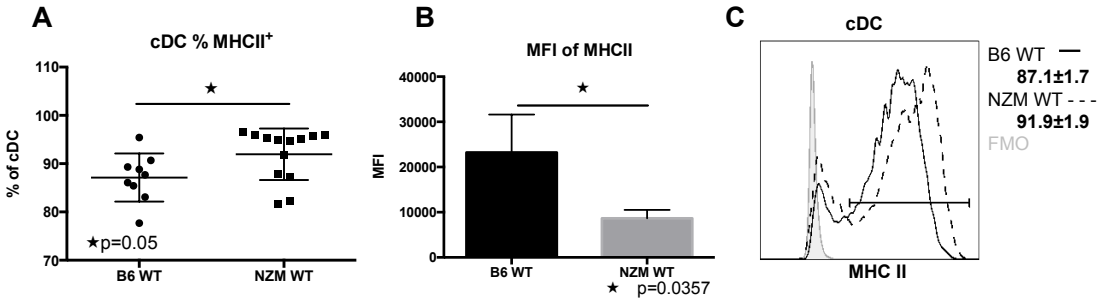


Figure 16. Frequency of activated spleen cDCs in B6 and NZM mice. Frequency of MHCII+ cDCs **(A)** and the MFI of MHCII+ cDCs **(B)** in the spleens of B6 WT (n=9) and NZM WT (n=13) 10-14 week old female mice in ≥ 4 separate experiments. Representative flow cytometry plot **(C)** Gates for frequency were set in each experiment based on a fluorescence minus one (FMO) control. Results are expressed as the mean ±SD. Statistical analysis performed with a student's T test analysis.

However, each cDC expresses less of the MHCII molecule. This finding suggests that NZM cDCs may be down regulating MHCII as either a negative feedback mechanism or as part of disease pathogenesis.

Absence of functional ERα does not alter the maturation state of pDCs in NZM2410 mice.

Since ERα impacts cell number in the bone marrow and is known to alter DC development *in vitro*, we wanted to ensure the pDCs from ERαKO mice reached maturity, as lack of full maturity may explain an impaired ability to activate. We measured the expression of Ly49Q on the spleen pDC population as a marker of pDC maturation. Ly49Q is only expressed on fully mature pDCs and denotes their ability to respond to TLR stimuli and produce IFNα [73, 74, 83]. In B6 mice, the majority of spleen pDCs express this marker. The majority of spleen pDCs from NZM female mice also expressed Ly49Q and ERα deficiency did not alter the frequency or absolute number

of pDCs expressing Ly49Q (Figure 17). ERα deficiency thus does not alter the maturation status of pDCs in the spleen.

Figure 17

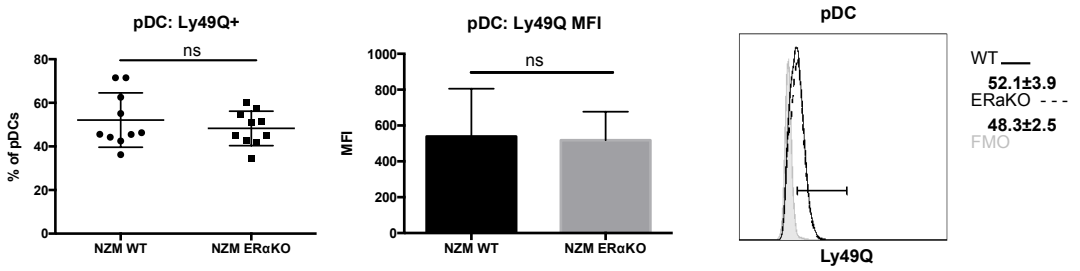


Figure 17. Frequency of Ly49Q$^+$ spleen pDCs. Frequency of Ly49Q$^+$ pDCs and MFI of Ly49Q on pDCs in the spleens of WT (n=10) and ERαKO (n=10) 10-14 week old female NZM2410 mice in 4 separate experiments Results are expressed as the mean ±SD. Statistical analysis performed with a t-test analysis (A,B) or Mann-Whitney analysis (C).

Absence of functional ERα reduced the frequency of PDC-TREM+ pDCs in NZM2410 mice.

Since the reduced frequency of MHCII$^+$ pDCs was not detected in B6 mice, we hypothesized the ERα modulates pDC response to inflammatory stimuli present in disease, such as TLR 7 or 9 ligands. In lupus, self-nucleic acids in immune complexes stimulate TLR7 and 9 in pDCs [91]. TLR signaling results in the activation of pDCs. Although we studied mice pre development of autoantibodies and renal disease, it is likely some immune abnormalities are present in the preclinical state of disease. To determine if altered TLR signaling may be responsible for the reduction in pDC MHCII expression, we measured the expression of the early pDC activation marker PDC-TREM. PDC-TREM is expressed on pDCs preferentially after exposure to TLR ligands and type

I IFNs [84]. Absence of functional ERα significantly and reproducibly reduced the frequency of PDC-TREM^{+} pDCs in the spleens of NZM mice *in vivo* (Figure 18). pDCs from NZM mice also had reduced MFI of PDC-TREM. This reduction was also specific to the NZM mice, as it was not detected in pDCs from ERαKO B6 mice (Figure 19). The percent of splenic pDCs expressing PDC-TREM was significantly higher in the WT NZM mice versus B6 mice suggesting heightened pDC activity in lupus prone mice even at the preclinical disease stage (Figure 20). This finding suggests the alteration in pDC activation state may be due to an impaired response to TLR ligands *in vivo* in ERαKO pDCs from NZM mice.

Figure 18

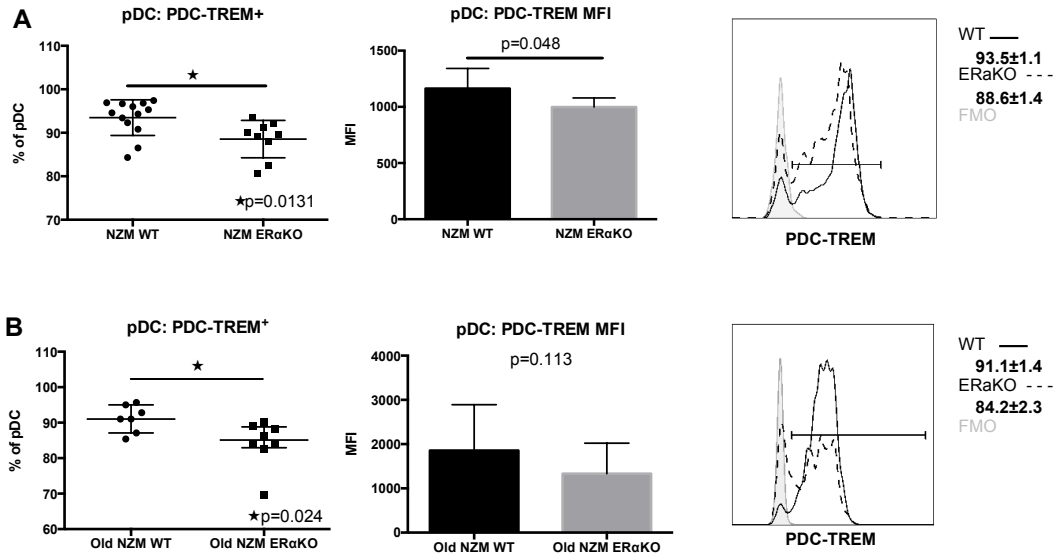


Figure 18. Frequency of PDC-TREM^{+} spleen pDCs. Frequency of PDC-TREM^{+} pDCs and MFI of PDC-TREM on pDCs in the spleens of WT (n=13) and ERαKO (n=9) 10-14 week old female NZM2410 mice in 6 separate experiments **(A)**. Frequency of PDC-TREM^{+} pDCs and MFI of PDC-TREM on pDCs in the spleens of WT (n=7) and ERαKO (n=8) 22-26 week old female NZM2410 mice in 3 separate experiments **(B)** Results are expressed as the mean ±SD (A) or median ±IQR. Statistical analysis performed with a t-test analysis (A) or Mann-Whitney analysis (B)

We also measured PDC-TREM expression on pDC from 22-26 week old female WT and ERαKO NZM mice. Like, the pre-disease mice the ERαKO had a reduced

frequency of PDC-TREM+ pDCs compared to WT pDCs (Figure 20). We did not see any changes in PDC-TREM+ pDC frequency with advanced age, suggesting this change in pDC activation status remains constant through the course of disease.

Figure 19

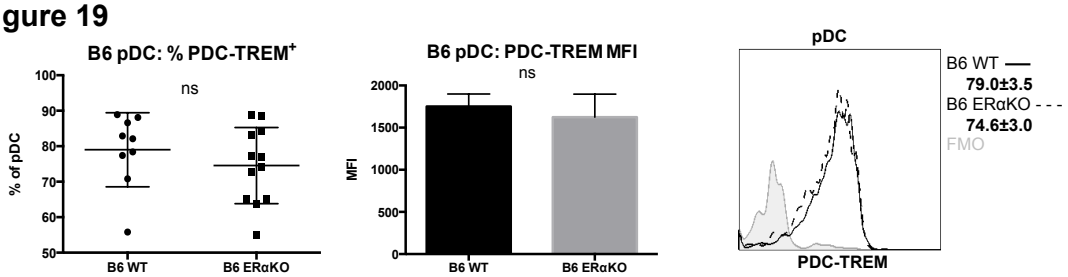


Figure 19. Frequency of PDC-TREM+ pDCs in the spleens B6 mice. Frequency of PDC-TREM+ pDCs and MFI of PDC-TREM on pDC in the spleens of WT (n=9) and ERαKO (n=12) 10-14 week old female B6 mice in 4 separate experiments. Gates for frequency were set in each experiment based on a fluorescence minus one (FMO) control. Results are expressed as the mean ±SD. Statistical analysis performed with a Student's T Test.

Figure 20

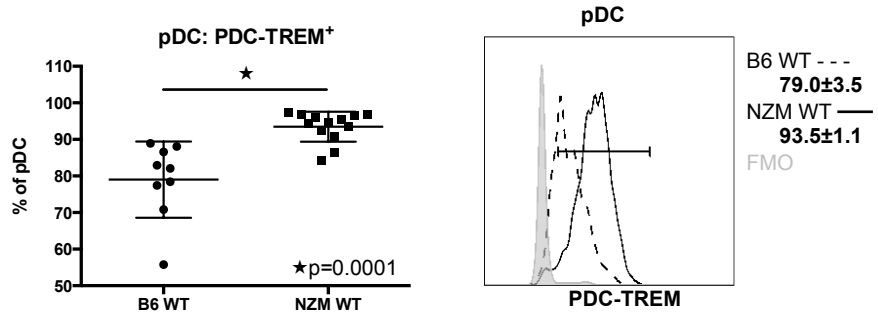


Figure 20. Frequency of PDC-TREM+ pDCs in the spleens of B6 and NZM mice. Frequency of PDC-TREM+ pDCs in the spleens of B6 WT (n=9) and NZM WT (n=13) 10-14 week old female mice in ≥ 4 separate experiments. Gates for frequency were set in each experiment based on a fluorescence minus one (FMO) control. Results are expressed as the mean ±SD. Statistical analysis performed with a Student's T test.

IFN signature is reduced in ERαKO NZM mice prior to clinical disease

PDC-TREM expression is required for pDCs to produce type I IFNs. Since ERα modulates PDC-TREM expression, we hypothesized it also impacts type I IFN activity in lupus prone mice. To assess type I IFN activity, we measured expression of type I IFN responsive/signature genes in the whole spleens of NZM mice. We measured genes that are elevated in BM derived DCs of pre-disease lupus-prone mice compared to B6 mice [47]. Using this previously validated assay, we found the absence of ERα significantly reduced the expression of the Type I IFN signature genes Cxcl-10 and Mx1 (Figure 21).

Figure 21

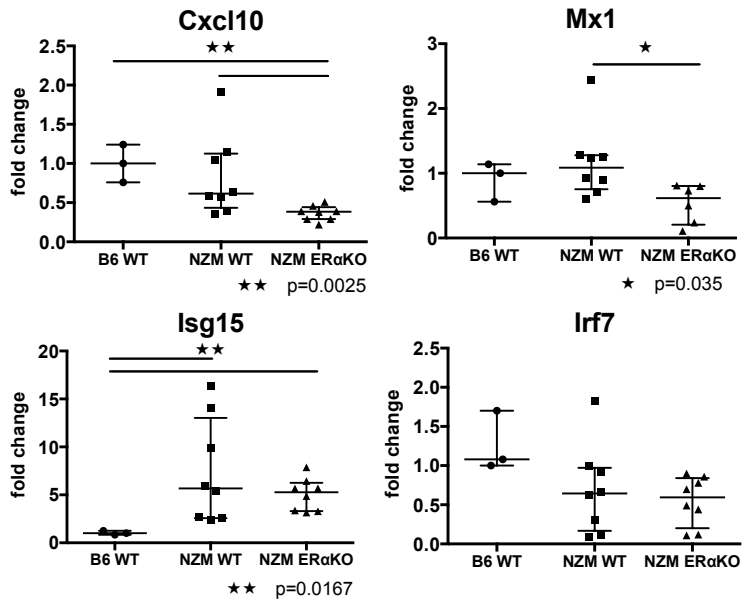


Figure 21. Expression of IFN signature genes in spleens of pre-disease NZM WT and ERαKO mice Expression of Cxcl10, Mx1, Isg15, and Irf7 in spleens of WT (n=10) and ERαKO (n=13) 10-14 week old female NZM mice in ≥ 4 separate experiments. Fold change was calculated compared to 13 week old female B6 mouse. Results are expressed as the median ±IQR. Statistical analysis performed with Kruskal-Wallis analysis.

There was a trend towards reduced Isg-15, and Irf-7 expression (Figure 21). There were no detectable levels of IFNα or IFNβ, which can be attributed to the relative scarcity of pDCs and cDCs in the spleen. Furthermore there was an increase in Isg-15 expression in

Figure 22

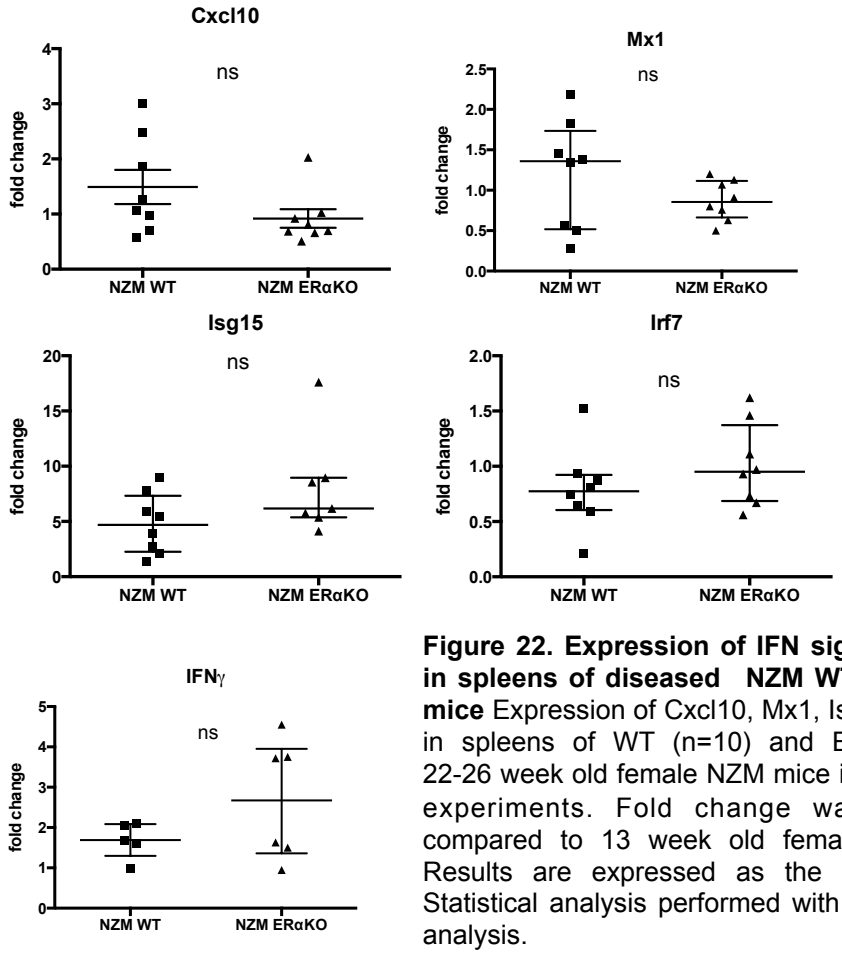


Figure 22. Expression of IFN signature genes in spleens of diseased NZM WT and ERαKO mice Expression of Cxcl10, Mx1, Isg15, Irf7, IFNγ in spleens of WT (n=10) and ERαKO (n=13) 22-26 week old female NZM mice in ≥ 4 separate experiments. Fold change was calculated compared to 13 week old female B6 mouse. Results are expressed as the median ±IQR. Statistical analysis performed with Kruskal-Wallis analysis.

NZM compared to B6 controls, suggesting IFN activity is increased in NZM mice compared to B6 controls. This finding demonstrated that ERα deficiency reduced type I IFN activity in pre-clinical disease NZM mice. We believe that the inflammatory state of NZM female mice, even prior to clinical disease, leads to detectable differences in pDC activation due to a key role of ERα in TLR/IFNα mediated inflammation. It is important to note, we did not detect an increase in all IFN signature gene expressions in the spleens from NZM mice compared to B6 controls. We chose these genes because they are increased in BMDCs from SLE1,2,3 mice compared to B6, however, they are not

consistently increased in ex vivo isolated spleen cells [47]. This is likely due to the heterogeneity of the spleen cells.

Figure 23

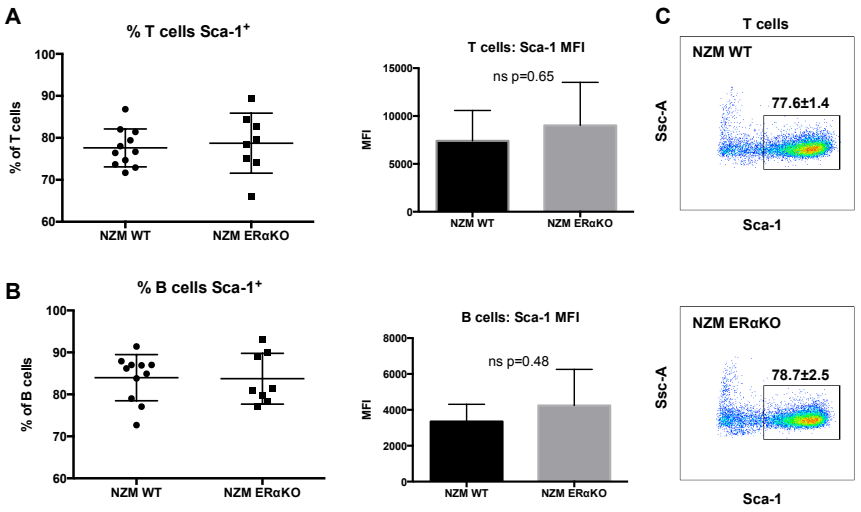


Figure 23. Frequency of Sca-1⁺ T and B cells in the spleen of NZM WT and ERαKO mice Frequency of Sca-1⁺ T cells DCs **(A)** and Sca-1⁺ B cells **(B)** in the spleens of WT (n=11) and ERαKO (n=8) 10-26 week old female mice in ≥ 4 separate experiments. Representative flow cytometry plot **(C)**. Results are expressed as the mean ±SD. Statistical analysis performed with Student's T Test analysis.

We also wanted to determine if advanced disease could expand the difference in IFN signature gene expression between WT and ERαKO NZM mice, therefore we measured the expression of the IFN signature genes in a 22-26 week old cohort of WT and ERαKO NZM mice. In these mice we did not see a significant change in any genes. Furthermore, there was a trend towards increased levels of mx1 and irf-7 in the spleens of NZM ERαKO mice compared to WT controls (Figure 22). The lack of change between IFN signature genes in the aged group of mice may be due to the immunological changes occurring from the disease process. Additionally, we measured IFNγ expression in the spleen, as this may contribute to IFN signature gene expression, thus accounting for differences between genotype. However, IFNγ does not likely contribute to the IFN signature as levels were not reduced in the spleens ERαKO compared to WT.

To strengthen our evidence for ERα impacting type I IFN activity in NZM mice, we chose to measure Sca-1 expression on spleen T and B cells, which is a measure of

chronic type I IFN exposure. We did not detect any difference in the frequency of B or T cells expression SCA-1 nor did we detect a change in MFI between the ERα genotypes (Figure 23). We performed this test in both old (22-26 week old) NZM and young (12-14 week old) NZM mice. The lack of change could be attributed to a low sensitivity to detect a subtle change caused by the deficiency of functional ERα.

We also measured IFN signature genes in the kidneys of diseased (22-26) week old NZM2410 mice. We found that 1 of the WT mice had highly increased levels of the IFN signature genes, suggesting ERα signaling plays a role in kidney IFN activity (Figure 24). This experiment was preformed only once because we could not detect pDCs in the kidneys of NZM mice, however, kidney endothelial cells may contribute to this IFN signature and thus could be explored further [92].

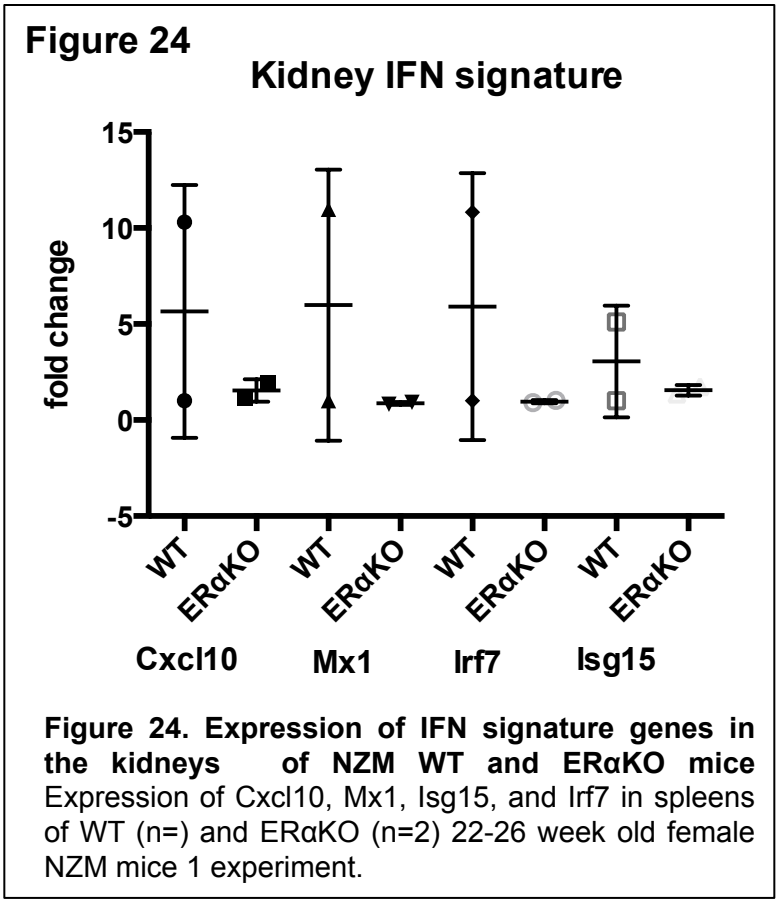


Figure 24. Expression of IFN signature genes in the kidneys of NZM WT and ERαKO mice Expression of Cxcl10, Mx1, Isg15, and Irf7 in spleens of WT (n=) and ERαKO (n=2) 22-26 week old female NZM mice 1 experiment.

We measured IFN signature in total spleen cells after 18 hours of TLR9 stimulation. We preformed this test to study the IFN signature producing capacity of the spleen DCs. Using this method we did not detect a difference in IFN

Figure 25

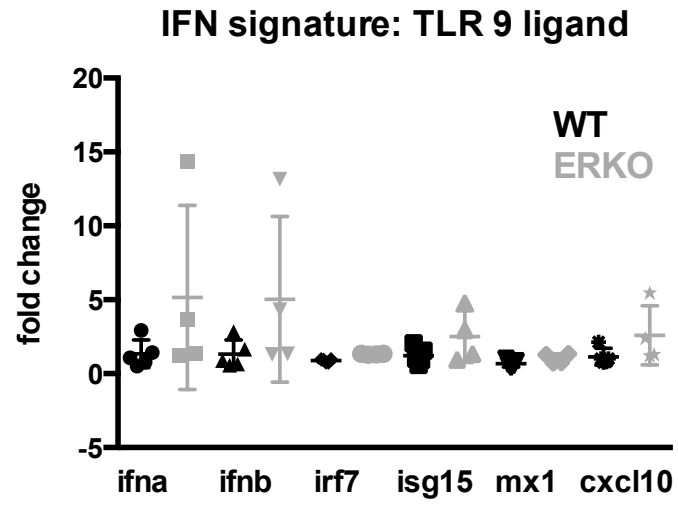


Figure 25. Expression of IFN signature genes in TLR9 stimulated spleens of NZM WT and ERαKO mice Expression of Ifna, Ifnb, Cxcl10, Mx1, Isg15, and Irf7 in spleens cells of WT (n=5) and ERαKO (n=5) 10-14 week old female NZM mice after 18 hours of TLR 9 sitmulation in ≥ 3 separate experiments. Results are expressed as the mean ±SD. Statistical analysis performed with Kruskal-Wallis analysis.

signature gene expression between genotypes (Figure 25).

Since PDC-TREM expression is consistently reduced in pDCs from ERαKO mice and PDC-TREM is an important regulator of pDC type I IFNα production, it is likely our measures of type I IFN activity were not sensitive enough to detect large change between genotypes. However, we did detect a reduction in two of the four IFN signature genes measured in the spleens of pre-disease NZM2410 mice, suggesting IFN activity is reduced in ERαKO mice. This reduction in IFN activity may contribute to reduced disease activity.

Summary

Ex vivo activation status

- Pre-disease NZM ERαKO mice have a reduced frequency of pDCs expressing MHCII and PDC-TREM compared to WT NZM mice
- NZM mice have an increased frequency of PDC-TREM expressing pDCs compared to B6
- In B6 mice, ERαKO does not alter PDC activation status
- Pre-disease NZM ERαKO have reduced expression of IFN signature genes in the spleen compared to NZM WT mice

3.4 Discussion

In pre-disease lupus prone NZM2410 mice, the functional mutant of ERα reduced pDC activation phenotype *in vivo* without impacting pDC number or maturation. Specifically, the functional mutant of ERα reduced pDC expression of PDC-TREM, a modulator of pDC type I IFN production. The absence of functional ERα simultaneously reduced type I IFN activity in the spleens of the pre-disease NZM mice. Given the key role of pDCs and type I IFNs in murine models of lupus, these findings suggest that ERα's ability to modulate pDC activation and type I IFN activity is a mechanism by which the functional mutation of ERα protects NZM mice from disease.

DCs from lupus patients and lupus prone mice have altered expression of activation markers [70, 87, 93]. Our study is unique in measuring the effect of ERα signaling on the *in vivo* pDC activation state in lupus prone mice without *ex vivo* stimulation of the pDCs. We have identified changes in pDC activation state induced by the absence of functional ERα signaling in NZM mice prior to the development of clinical disease, therefore, our findings may represent a mechanism by which ERα functional mutation protects NZM mice from disease rather than reflecting an effect of differential disease severity. Other studies have previously reported the impact of estrogen/ERα on DC activation after stimulation with TLR ligands. Our data is in agreement with Douin-Echinard et al. which showed that ERα deficiency reduced TLR9 mediated MHCII expression without altering CD40 expression in BMDCs cultured from B6 mice [12]. Alternatively, in Li et al. the addition of estradiol at the time of TLR9 stimulation increased pDC CD40 expression, suggesting estrogen differentially affects DC activation phenotype depending on whether the estrogen exposure/signaling is short

(*in vitro* addition of estrogen) or long-term *(in vivo* deficiency of ERα *)* [53]. The long-term deficiency of ERα signaling, both in our study and Douin-Echinard et al. demonstrated a specific impact on pDC MHCII expression in ERα functionally deficient cells [12]. Furthermore, the functional mutation of ERα reduced MHCII expression on spleen pDCs from NZM mice, but not spleen pDCs from B6 controls. Therefore, ERα 's role in pDC activation phenotype *in vivo* appears either strain specific or represents an effect of early disease in the NZM mice. One reason for this specificity could be that ERα modulates pDC activation in response to immune stimuli present uniquely in NZM mice (e.g. TLR7/9 ligands) [94]. Alternatively, a pDC intrinsic/genetic factor specific to NZM mice could mediate ERα's effect . We believe the former mechanism is most likely given that, after *in vitro* TLR stimulation, ERα signaling also increased expression of MHCII on pDCs from both NZM and B6 mice. These results suggest ERα's effect on the pDC activation phenotype, in response to TLR stimulation is independent of the pDC strain background. In this study we have both confirmed that the functional mutation of ERα impaired pDC activation phenotype and the reduction this occured in pDCs of NZM mice prior to development of clinical disease. These findings indicate pDC abnormalities in NZM2410 mice are an early marker of disease, which are impacted by ERα expression.

A novel finding of our results is that the functional mutation of ERα reduced PDC-TREM expression on spleen pDCs from pre-disease NZM mice. To our knowledge, this is the first report of an effect of estrogen and/or ERα on PDC-TREM expression. pDCs acquire PDC-TREM protein on the cell surface upon TLR stimulation and this surface expression is required for pDCs to produce IFNα [84]. The reduction in PDC-

TREM expression on pDCs from ERαKO NZM mice suggests that the absence of functional ERα impairs activation in response to TLR signaling *in vivo*. Wataria et al. showed that TLR7 and TLR9 ligands induced PDC-TREM expression and IFNα, TLR4 ligand, and PolyA stimulation alone could not induce PDC-TREM expression [84]. Since TLR7/9 stimuli are known to be present in WT and ERαKO NZM mice (immune complexes), but less so in B6 mice, *in vivo* pDC expression of PDC-TREM is likely mediated, at least partially, by TLR signaling [1]. However, Swiecki et al. showed that MyD88 was not required for PDC-TREM expression during HSV-1 infection, suggesting stimuli other than the MyD88 dependent TLR7 and 9 ligands can induce PDC-TREM expression [79]. Therefore ERα may alter PDC-TREM expression via another pDC activation pathway, such as NOD-like receptors or RIG-I-like receptor stimulation or more specific modulators of pDC activity, such as Ly49Q or SiglecH signaling *in vivo*.

The reduction in PDC-TREM expression on pDCs from NZM mice also suggests ERα modulates the pDC type I IFN production pathway. This idea is supported by the reduction in spleen type I IFN activity in NZM ERαKO mice. Our findings are in agreement with Seillet et al., who showed that estradiol via ERα enhanced pDC IFN production [16]. In Seillet et al., the deficiency of ERα prevented the estrogen mediated pre-programming of pDCs *in vivo,* thereby reducing pDC IFN production in response to TLR stimulation *[16]*. Our findings demonstrate a possible effector molecule through which ERα modulates pDC type I IFN production. Like MHCII expression, the functional mutation of ERα reduced PDC-TREM expression on pDCs from cultured BMDCs after *in vitro* TLR stimulation in both BMDCs from NZM and B6 mice. Since, after *in vitro* TLR stimulation, ERα deficiency impacts PDC-TREM expression in both

strains of mice, these data suggest ERα modulation of PDC-TREM may represent both a mechanism of disease protection and a general mechanism by which ERα impacts type I IFN production and TLR responses.

To accurately measure type I IFN activity in lupus prone mice, without artificially stimulating the cells, we measured the expression of type I IFN signature genes in the spleen. The genes measured are elevated in BMDCs and certain splenocytes of pre-disease *Sle*-1,2,3 mice compared to B6 mice [47]. Expression of these genes represents the cellular response to type I IFN stimulation, thereby the assay measures both the amount of functional type I IFN present in an environment and the ability of the affected cell to respond to type I IFN. We measured the IFN signature genes in total spleen cells, thus we measured the responsiveness of total spleen to type IFN. As previously published, IFN signature gene expression varies between spleen cell type, thus, this variation likely impaired our ability to detect dramatic changes in IFN signature gene levels. However, we did detect significant change in 2 of the 4 genes measured, suggesting Erα signaling positively impacts the cycle of type I IFN production and response. Based on the mechanics of the assay, ERα may alter the amount of type I IFN produced, the responsiveness of spleen cells to the IFN, or both.

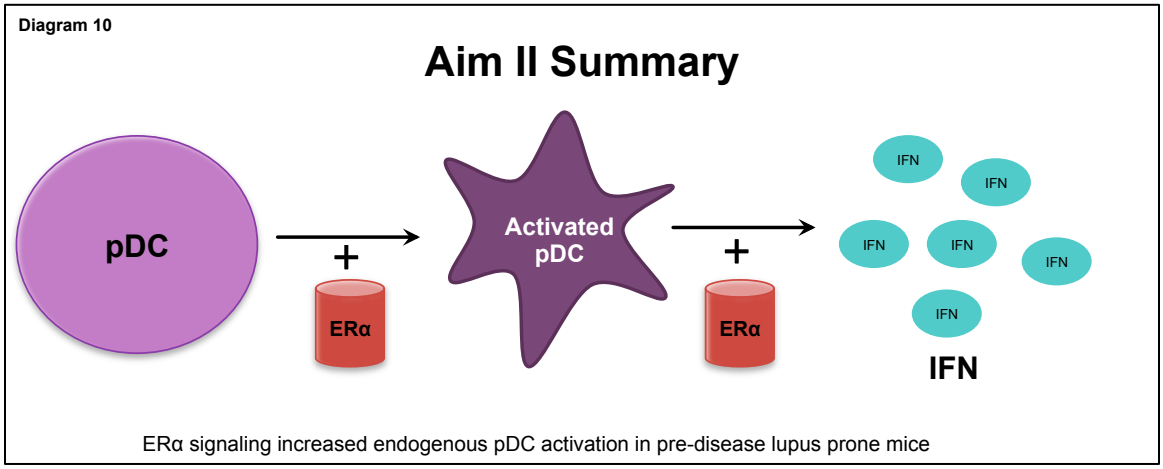
We hypothesized the difference in IFN signature gene expression between genotypes would widen as diseased progressed. However, the difference between the genotypes was reduced in diseased NZM mice. This finding may be due to immune abnormalities occurring as disease progresses or ERα signaling impacting a negative feedback loop. There was a trend towards increased ISG-15 in the spleens of the 22-26

week of NZM ERαKO mice compared to NZM WT mice. ISG-15 protein is a ubiquitin like protein that covalently attaches to target proteins [95]. Once attached ISG-15 can regulate protein function and survival. Thus, it may be possible increased levels of ISG-15 in the ERαKO negatively regulates type I IFN production as disease progresses.

Other studies have shown estrogen via ERα affects pDC production of type I IFN [16, 53]. In Seillet et al., the treatment of post menopausal women with estradiol *in vivo* induced pDCs to produce higher amounts of IFNα upon TLR stimulation [16]. Further more, Seillet et al. showed that estradiol's impact on IFNα production was dependent on ERα in mice. Li et al. demonstrated that simultaneous *in vitro* estradiol and TLR treatment enhanced pDC type I IFN production compared to TLR stimulation alone [53]. Although these papers argue that estradiol/ERα alters pDC IFN production through different mechanisms, they both agree that estradiol/ERα positively impacts type I IFN production, which may explain our findings. Although these studies address similar questions as our work, they differ in that they focus strictly on pDC production of type I IFNs in response to acute exposure to TLR ligand. Since the IFN signature gene expression measures total type I IFN activity in a given environment, our data represents type I IFNs produced by pDCs in response to endogenous stimuli present in the spleens of lupus prone mice. This is an important differentiation, as TLR stimulation does not fully account for the IFN signature seen in lupus prone mice [47]. Alternatively, a different cell type could produce the IFN. Although pDCs are typically the major source of type I IFN, viral infection can induce cDCs to produce significant amounts of type I IFN [96]. Therefore, in the future, both pDC and cDC type I IFN production should also be measured in the lupus prone mice.

3.5 Conclusions

In pre-disease NZM2410 mice, ERα signaling positively impacted pDC activation state. Since this finding is restricted to NZM2410 mice and not seen in B6 mice, ERα signaling likely impacted pDC activation in response to disease specific stimuli. Furthermore, ERα signaling positively impacted pDC expression of PDC-TREM, a regulator of pDC IFNα production. ERα also positively impacted endogenous IFN activity in NZM mice prior to the development of clinical disease. These findings suggest ERα signaling may alter the IFN production pathway by influencing expression of PDC-TREM. Since type I IFNs are critical in lupus pathogenesis, we believe ERα's impact on pDC and IFN activity may represent a mechanism by which the functional mutation of ERα protects mice from disease. Additionally, we found these changes prior to the development of clinical disease, which further supports the hypothesis that these changes impact pathogenesis.



3.6 Future Directions

Future work could address ERα's role on *in vivo* type I IFN production. Although we detected a change in total spleen IFN signature gene expression in the NZM ERαKO mice we did not identify the cells producing IFN nor did we identify the cells affected by IFN. To answer these questions we could sort pDCs and cDCs from WT and ERαKO NZM mice. Then IFNα and IFNβ transcripts could be measured both with and without exogenous TLR stimulation. This would determine if ERα regulates pDC IFN production *in vivo*. Additionally, we can sort B, T and NK cells from the spleen and measure their expression of IFN signature genes to determine if ERα signaling impacts their response to endogenous IFN activity.

We looked for pDCs in the kidneys in NZM mice. However, were not able to detect these cells by flow cytometry. Other groups have reported a lack of kidney pDCs in mice, however pDCs are present in the kidneys in humans with SLE [79]. Additionally, we detected highly increased expression of IFN signatures genes in 1 WT NZM mouse, but not the 2 ERαKO mice. Therefore it may be worthwhile to look into pDCs and type I IFNs in the kidneys of WT and ERαKO NZM mice. Since resident kidney cells produce type I IFNs in lupus, ERα may regulate kidney resident cell production of IFN [92]. Alternatively, it is possible that pDCs infiltrate the kidney and murine lupus, but lose traditional pDC markers. pDC markers can be lost *in vitro* after TLR stimulation [78]. Thus, a lupus mouse model with a fluorescently tagged pDCs may help determine the fate of pDCs as disease progresses. Lastly, ERα may regulate cDC production of type I IFNs in the kidneys. cDCs are found in lupus kidneys and cDCs have been shown to be able to produce large amounts of type I IFNs under inflammatory

conditions [Cunningham, unpublished data] [96]. Therefore, IFN production by kidney cDCs should be investigated.

Chapter 4: The impact of estrogen receptor α signaling on PDC-TREM expression

Chapter 4: The impact of estrogen receptor α signaling on PDC-TREM expression

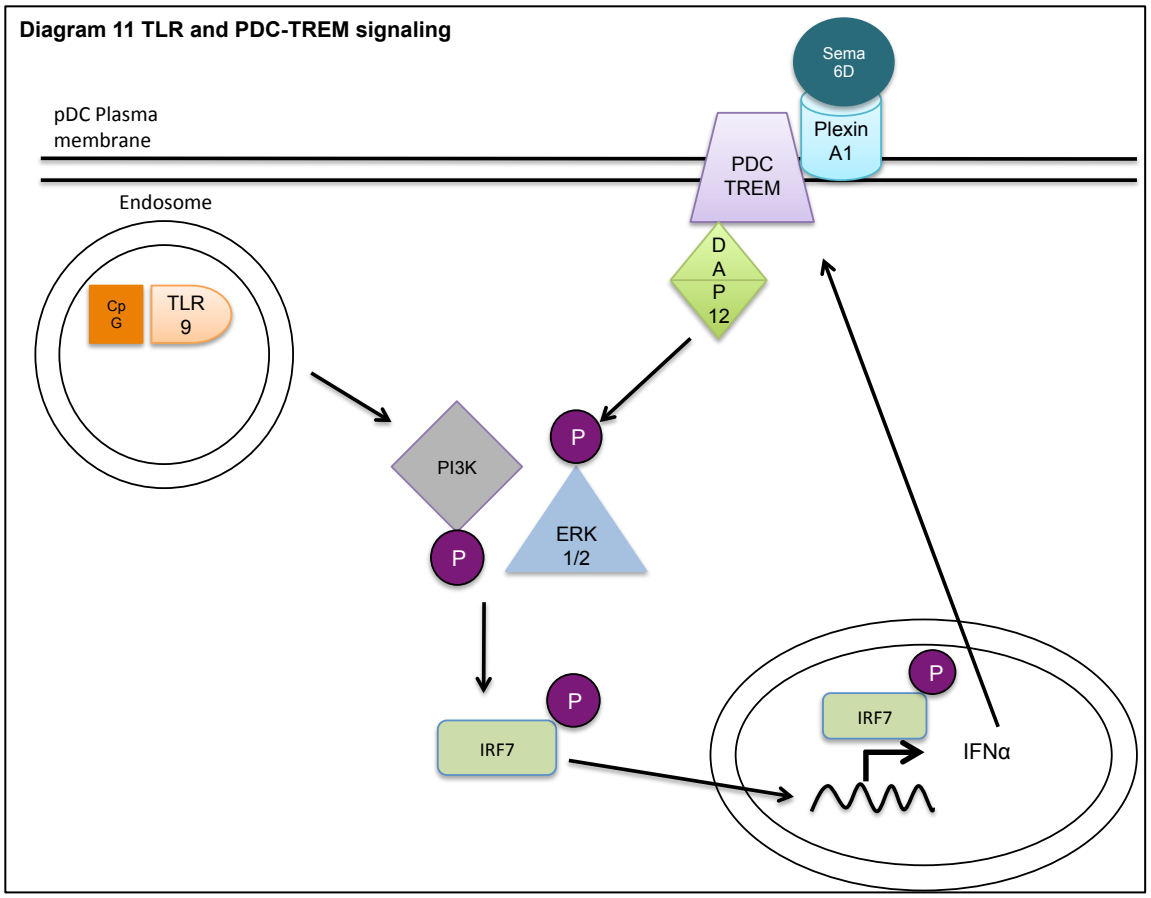
4.1 Introduction

Pre-disease ERαKO NZM mice have a reduced frequency of PDC-TREM^{+} pDCs compared to WT NZM mice. PDC-TREM regulates pDC IFN production, thus, likely impacts disease activity in NZM mice. In this chapter, we will explore the mechanisms by which ERα signaling mediates expression of PDC-TREM.

PDC-TREM

TREM, triggering receptor expressed on myeloid cells, proteins are a family of surface receptors that function to modulate cellular responses on myeloid cells [97]. TREM proteins are expressed on dendritic cells, macrophages, osteoclasts, microglia, monocytes, and platelets [97]. Both human and mouse cells express TREMs [97]. In mice, TREMs 1-4 have been identified and in humans TREM proteins 1 and 2 have been identified, however, a genomic region with similarities to mouse TREM 3 and 4 are present [97]. TREMs function to integrate cell signals and regulate cell responses through both inhibitory and activating mechanisms [97]. TREMs signal through the adaptor molecule DAP12, which may contain either an ITAM or ITIM, with activating or inhibiting functions, respectively [97]

PDC-TREM, also known as TREM-4, is the most recently described TREM protein in mice [97]. It is expressed exclusively on pDCs and is expressed after TLR stimulation [84]. It is not detected on naive pDC. Its surface expression, in response to TLR stimulation, is dependent on MyD88, IFNAR, and DAP12 [84]. Once expressed on



the cell surface PDC-TREM associates with PlexinA1 and the intracellular adaptor molecule DAP12, which contains an ITAM (activation) signaling domain [84]. Although MyD88 is required for PDC-TREM expression induced by TLR stimulation, during HSV1 infection PDC-TREM is expressed independent of MyD88, suggesting non-MyD88 dependent stimuli may contribute to its surface expression [79]. Although little is known about PDC-TREM ligands, PDC-TREM associates with PlexinA1. After sub optimal CpG treatment, treatment with the PlexinA1 ligand, sema6D treatment induces IFNα production [84]. The concurrent stimulation with sem6D and suboptimal CpG induces PI3K, ERK1/2, and IKKα/β phosphylation [84]. These signaling molecules are involved in IFNα production (Scheme 6). PDC-TREM is required for CpG induced IFNα production in Flt3L derived mouse pDCs. pDCs treated with shRNA for pdc-trem,

produce significantly less IFNα, but continue to produce equal levels of IL-12, IL-6, and TNFα [84].

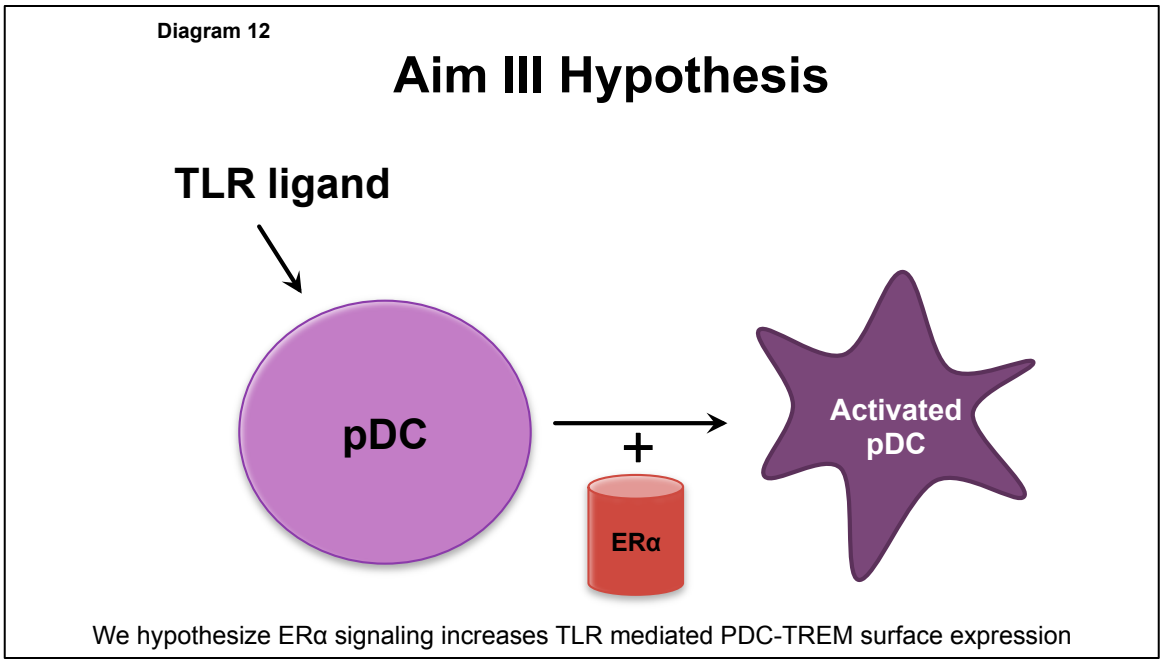
Mammalian target of rapamycin

The mammalian target of rapamycin (mTOR) signaling promotes pDC production of type I IFN [82, 98]. The mTOR signaling cascade is activated by phosphatidylinositide 3-kinase (PI3K). When PI3K is activated by phosphorylation, it activates mTOR, which results in the phosphorylation and nuclear localization of IRF7, a critical step in pDC type I IFN production. Both TLR and PDC-TREM signaling phosphorylate PI3K, thus both may promote mTOR activation [84, 99, 100].

Additionally, PI3K is a known target of ERα [99, 101, 102]. In epithelial and endothelial cells, ERα binds to the p85alpha regulatory subunit of PI3K and induces activation of PI3K and mTOR. Furthermore, the mTOR pathway is overactive in SLE. In mice with lupus nephritis, glomerular PI3K and mTOR activity were increased compared to healthy controls[103]. Human and murine lupus are improved by rapamycin treatment [104-106].

4.2 Hypothesis

pDC expression of PDC-TREM is required for IFNα production, a key cytokine in lupus pathogenesis. Pre-disease ERαKO NZM mice have reduced frequencies of PDC-TREM^{+} pDCs compared to pre-disease WT NZM mice, suggesting ERα signaling impacts PDC-TREM expression. Additionally NZM mice have higher frequencies of PDC-TREM^{+} pDCs compared to B6 control mice, thus expression of PDC-TREM is associated with disease. Furthermore, ERαKO does not impact PDC-TREM levels in B6 mice, suggesting ERα's impact on PDC-TREM expression occurs in response to disease specific stimuli. One such stimuli is lupus immune complexes which, stimulate TLR7/9 in pDCs. **I hypothesized ERα signaling promotes TLR mediated PDC-TREM surface expression and this promotion occurs through an estradiol mediated increase in pdc-trem RNA expression.**



4.3 Results

ERα signaling impacts TLR mediated induction of PDC-TREM surface expression on BM derived pDCs from NZM mice

To determine if the absence of functional ERα signaling impairs PDC-TREM expression in response to TLR stimulation, we treated BMDCs from Flt3L driven cultures, containing a mixture of pDCs and cDCs, from WT and ERαKO NZM mice with the TLR 9 ligand, CpG DNA, and measured pDC activation. We first confirmed that PDC-TREM surface expression is increased 12 and 18 hours after TLR9 stimulation (Figure 26). Based on this finding we measured PDC-TREM expression after 18 hours of TLR9 stimulation for the remainder of experiments. As shown in Figure 27A, ERα deficiency reduced the frequency of pDCs expressing PDC-TREM. In the same cultures, ERα deficiency also reduced the frequency of MHCII expression on both pDCs and cDCs following TLR 9 stimulation (Figure 28). There was no induction of PDC-TREM in unstimulated controls (Figure 27B). To determine if this finding correlated with a reduction in inflammatory cytokine production we measured levels of Il-6, TNF-α, and Il-12p40 in the supernatant. We did not see a change in levels of any of these cytokines between genotypes after 18 hours of TLR 9 stimulation. (Figure 29) This finding

Figure 26

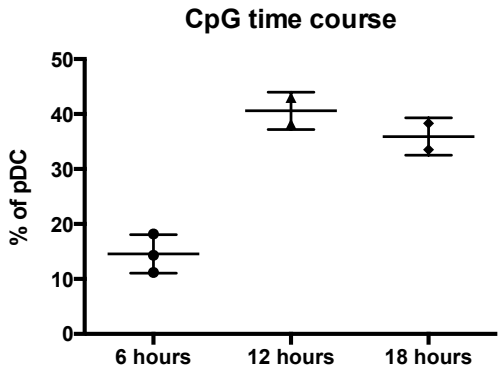


Figure 26. PDC-TREM surface expression on BM derived pDCs after TLR9 stimulation. BMDCs from WT NZM mice were treated with TLR9 agonist (CpG 1ug/ mL) for 6, 12, and 18 hours. PDC-TREM expression was measured by flow cytometry.

Figure 27

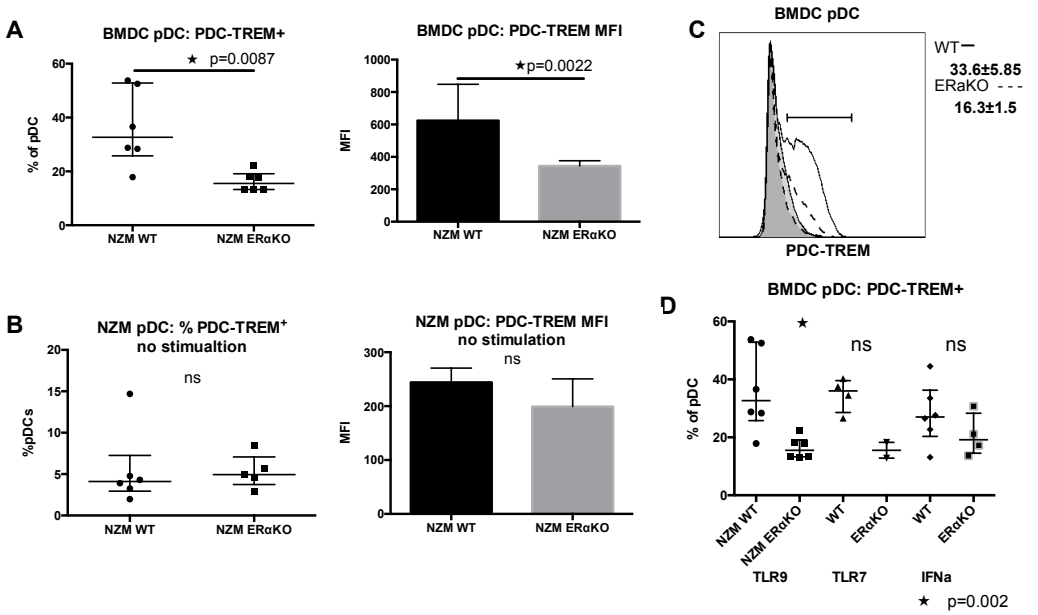


Figure 27. TLR ligand induced PDC-TREM expression on BM derived DCs from NZM2410 mice. BM from WT (n=6) and ERαKO (n=6) 10-14 week old female NZM2410 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and the entire mixture of pDCs and cDCs were plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) for 18 hours. Frequency of PDC-TREM$^+$ pDCs (CD11c$^+$B220$^+$) and MFI of PDC-TREM on pDCs **(A)**. Frequency of PDC-TREM$^+$ pDCs and MFI of PDC-TREM on pDCs treated with PBS control **(B)**. Representative flow cytometry, shaded histograms represent PBS control **(C)**. Frequency of PDC-TREM$^+$ pDCs from WT and ERαKO NZM mice after TLR9, TLR 7 (loroxibine 200ug/mL), and IFNα 100IU stimulation **(D)**. Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

suggestd the change in activation status does not correlate with a change cytokine production. Alternatively, our culture system consists of a mixture of pDCs, mDCs, and LDCs, which may dilute any change in cytokine production cause by one cell type. To address this issue, we sorted a pure population of Flt3L derived pDCs from B6 WT and ERαKO to measure TLR induced cytokine production. At 5 and 18 hours we did not detect a difference in Il-6, TNF-α, and Il-12p40 production between the genotypes (Figure 32). This finding suggests ERα signaling does not impact TLR mediated production of these cytokines.

Since PDC-TREM surface expression can also be induced by TLR 7 stimulation and its expression is dependent on type I IFN signaling, we stimulated BM derived DCs

Figure 28

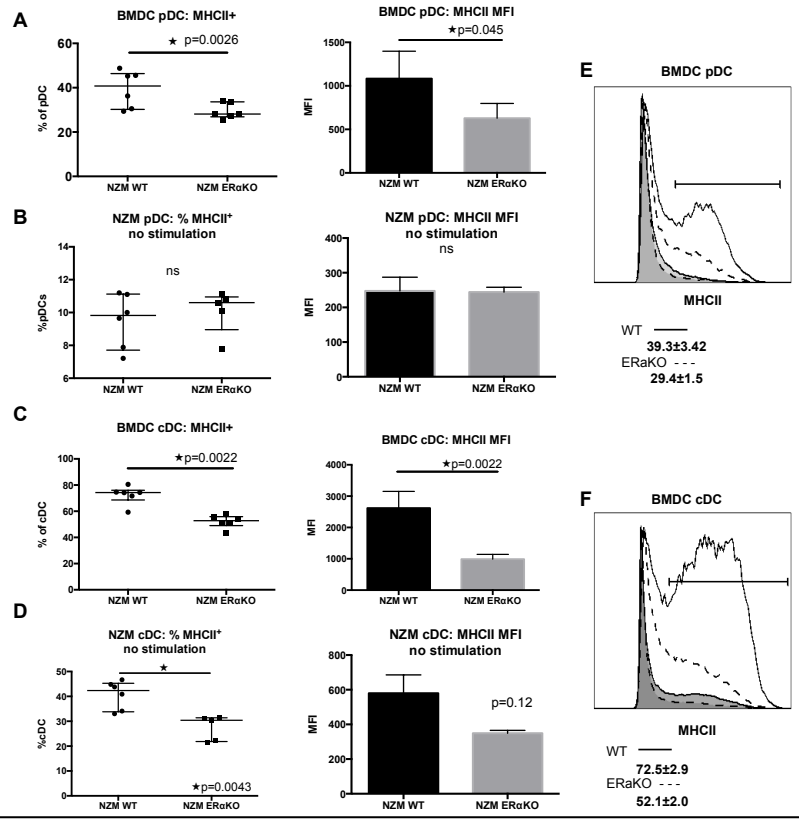


Figure 28. TLR ligand induced MHCII expression on BM derived DCs from NZM2410 mice. BM from WT (n=6) and ERαKO (n=6) 10-14 week old female NZM2410 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and the entire mixture of pDCs and cDCs were plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) for 18 hours. Frequency of MHCII$^+$ pDCs (CD11c$^+$B220$^+$) and MFI of MHCII on pDCs **(A)**. Frequency of MHCII$^+$ pDCs and MFI of MHCII on pDCs treated with PBS control **(B)**. Frequency of MHCII$^+$ cDCs (CD11c$^+$B220$^-$) and MFI of MHCII on cDCs **(C)**. Frequency of MHCII$^+$ cDCs and MFI of MHCII on cDCs treated with PBS control **(D)**. Representative flow cytometry, shaded histograms represent PBS control **(E,F)** Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

Figure 29

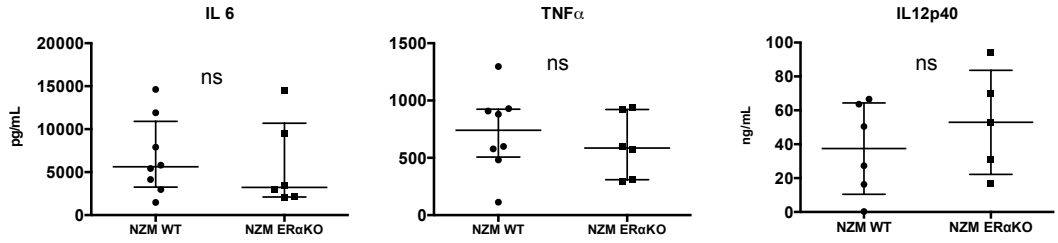


Figure 29. TLR ligand induced cytokine production by BM derived DCs from NZM2410 mice. BM from WT (n=8) and ERαKO (n=6) 10-14 week old female NZM2410 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and the entire mixture of pDCs and cDCs were plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) for 18 hours. Production of IL-6, TNF-alpha, and IL12p40 were measued by ELISA. Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

with TLR 7 ligand (Loroxibine) and IFNα and measured PDC-TREM surface expression. We found a trend towards a reduced frequency of PDC-TREM+ pDCs after TLR 7 stimulation and no difference after IFNα stimulation (Figure 27D). This finding

suggests ERα impacts both TLR7 and 9 mediated PDC-TREM expression. However, ERα does not greatly impact IFNα mediated PDC-TREM expression.

ERα signaling impacts PDC-TREM expression on BM derived pDCs from B6 mice.

To determine if ERα's effect on PDC-TREM expression was strain/disease specific we sorted pDCs (live, $CD11c^{+}B220^{+}CD11b^{-}$) from Flt3L driven BMDCs from WT and ERαKO B6 mice that were age and sex matched with the NZM mice and stimulated the pDCs with the TLR9 ligand, CpG DNA.

Figure 30

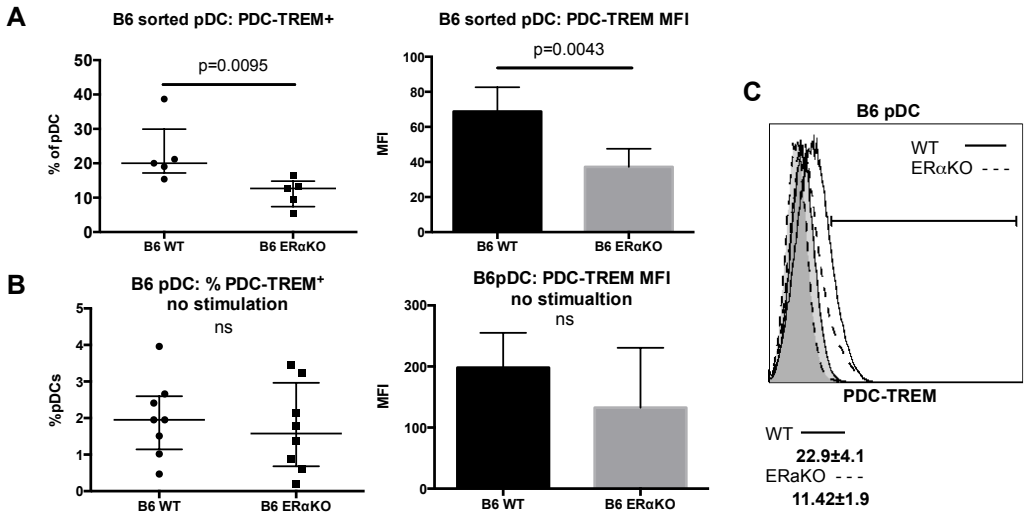


Figure 30. TLR 9 ligand induced PDC-TREM expression on BM derived pDCs from B6 mice. BM from WT (n=5) and ERαKO (n=6) 10-14 week old female C57/BL6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and pDCs ($CD11c^{+}$, $B220^{+}$, $CD11b^{-}$) were sorted using flow cytometry and plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) or PBS control for 18 hours. Frequency of PDC-TREM^{+} pDCs and MFI of PDC-TREM on pDCs **(A)**. Frequency of PDC-TREM^{+} pDCs and MFI of PDC-TREM on pDCs treated with PBS control **(B)**. Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Flow cytometry plots are representative of data in dot plots and shaded histograms represent PBS treated controls for each genotype **(C)**. Data obtained in 3 separate experiments.

In this pure pDC population, ERα deficiency also reduced PDC-TREM and MHCII expression indicating that the reduction of these activation markers on pDCs is both cell

intrinsic not dependent on changes in the pDCs caused by the disease state. (Figure 30 and 31). Therefore the reduction in PDC-TREM expression may represent a conserved mechanism by which ERα impacts pDC activation and may be applicable to the regulation of pDC in other models.

Figure 31

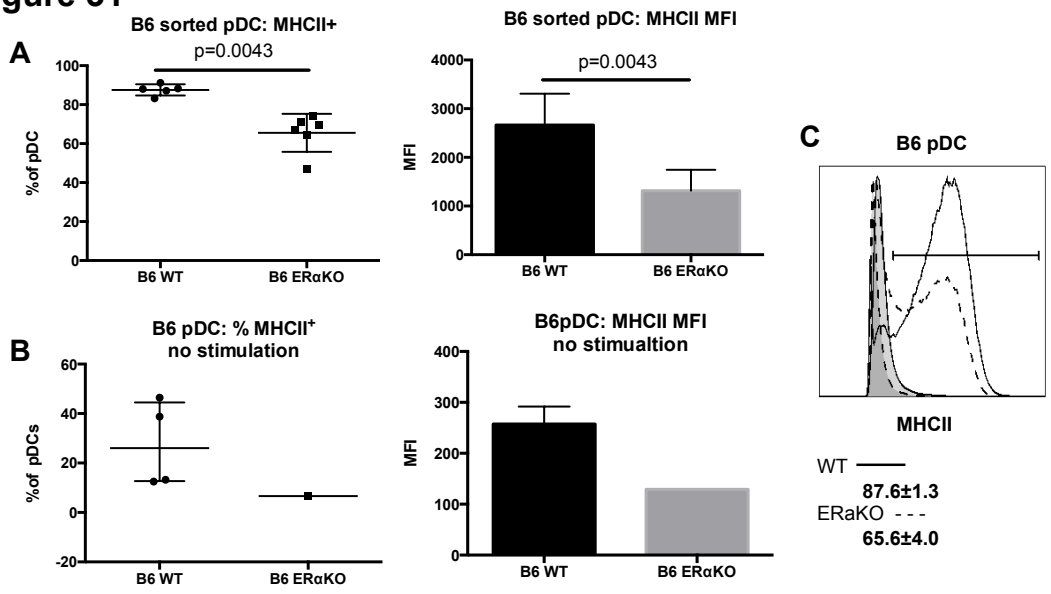


Figure 31. TLR 9 ligand induced MHCII expression on BM derived pDCs from B6 mice. BM from WT (n=5) and ERαKO (n=6) 10-14 week old female C57/BL6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and pDCs ($CD11c^+$, $B220^+$, $CD11b^-$) were sorted using flow cytometry and plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) or PBS control for 18 hours. Frequency of $MHCII^+$ pDCs and MFI of MCHII on pDCs **(A)**. Frequency of $MHCII^+$ pDCs and MFI of MHCII on pDCs treated with PBS control **(B)**. Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Flow cytometry plots are representative of data in dot plots and shaded histograms represent PBS treated controls for each genotype. Data obtained in 3 separate experiments.

MTOR pathway activation is increased in ERαKO pDCs after TLR stimulation

Since ERα signaling positively impacts PDC-TREM expression on pDCs, and

Figure 32

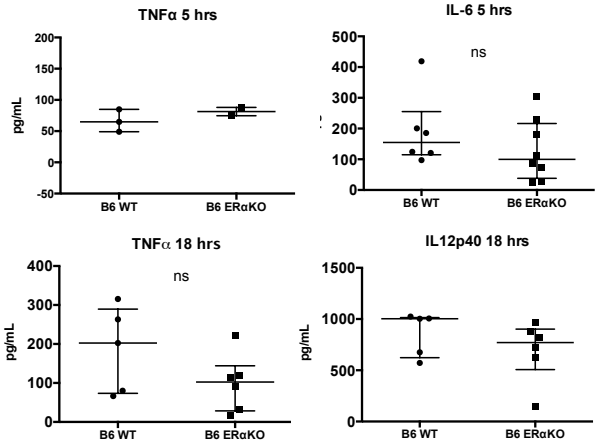


Figure 32. TLR ligand induced cytokine production by BM derived pDCs from B6 mice. BM from WT (n≥6) and ERαKO (n≥6) 10-14 week old female B6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and pDCs ($CD11c^+$, $B220^+$) were sorted using flow cytometry and plated at a concentration of 10^5 cells/mL and stimulated with 1ug/mL of CpG (TLR9 ligand) for 5 hours. For 18 hour stimulation the pDCs were plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand). IL-6 and TNF-alpha were measured at 5 hours and TNF-alpha and IL12p40 were measured at 18 hours. **(B)**. Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

Figure 33

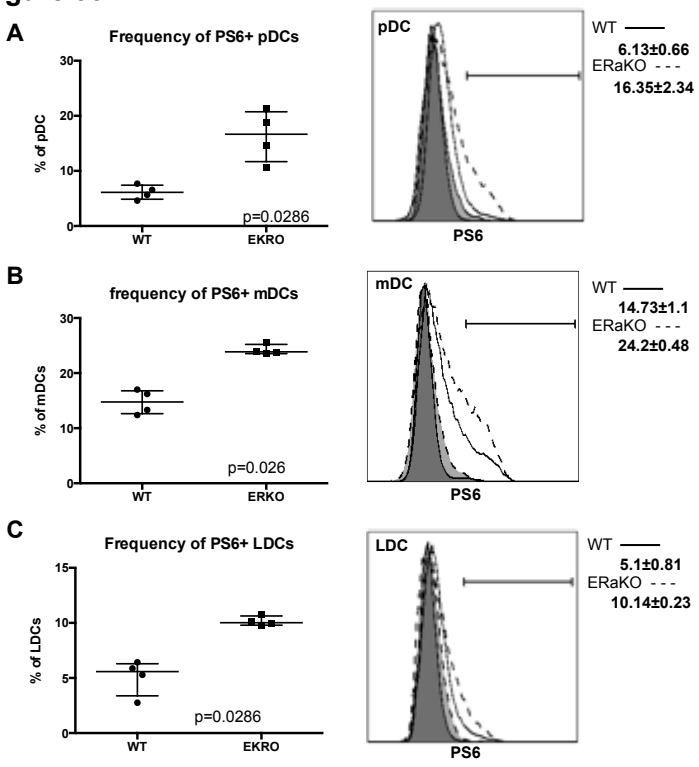


Figure 33. TLR 9 ligand induced PS6 expression on BM derived pDCs from NZM mice. BM from WT (n=4) and ERαKO (n=4) 10-14 week old female NZM2410 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. One day 7 cells were harvested and plated at a concentration of 2 million/ mL and stimulated with 1ug of CpG (TLR9 ligand) or PBS control for 18 hours. Frequency of $PS6^+$ pDCs **(A)**, $PS6^+$ mDCs **(B),** and $PS6^+$ LDCs **(C).** Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Flow cytometry plots are representative of data in dot plots and shaded histograms represent PBS treated controls for each genotype. Data obtained in 2 separate experiments.

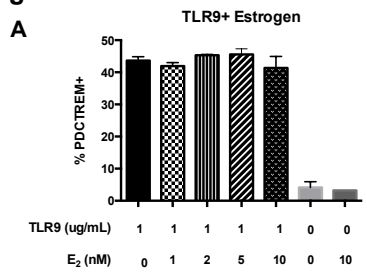
PDC-TREM impacts impacts PI3K phosphorylation, an activator of the mTOR pathway,

we hypothesized ERα signaling would also positively impact TLR mediated mTOR pathway activity. To test this hypothesis we measured mTOR activity via levels of phosphorylated S6 kinase in WT and ERαKO pDCs 18 hours after TLR 9 stimulation. We found that ERαKO pDCs expressed more phosphorylated S6, indicating that the mTOR pathway is more active in ERαKO pDCs and mDCs compared to WT after TLR stimulation. We hypothesized this finding may be due to a delayed onset of mTOR pathway activation in ERαKO. We measured mTOR activation in WT and ERαKO pDCs at short time points, 15 and 30 mins post TLR stimulation. At these time points, pDCs do not express phosphorylated S6. However, mDCs do expression phosphorylated 6S, indicating this pathway is more active in the mDCs compared to pDCs. Additionally, mDCs at 18 hours express more PS6 compared to pDCs, also suggesting this pathway is more important in ERα signaling in mDCs compared to pDCs.

Estradiol treatment does not impact TLR mediated PDC-TREM expression

Since ERαKO pDCs expressed lower levels of PDC-TREM after TLR activation, we hypothesized estradiol treatment may increase TLR mediated PDC-TREM expression on pDCs. To test this hypothesis, we treated BM derived $ER\alpha^{+/+}$ pDCs with 0, 1, 2, 5, and 10nM of estradiol at the time of TLR 9 stimulation (1ug/mL). We did not detect a change in pDC-TREM expression with any concentration of estradiol treatment (Figure 34A). This finding indicates that at an optimal level of TLR9 stimulation (1ug/mL) increasing estradiol levels do not impact PDC-TREM surface expression. Additionally, the addition of estradiol alone, without TLR stimulation, does not induce the surface expression of

Figure 34



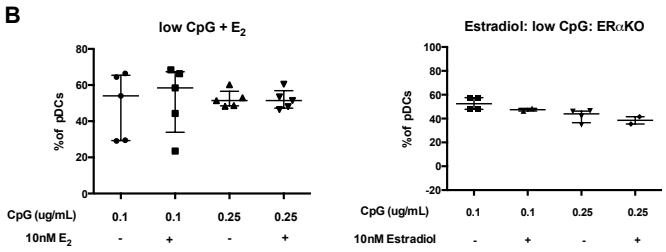


Figure 34. The effect of estradiol treatment on TLR 9 ligand stimulated BM derived pDCs. BM from WT and ERαKO mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. On day 7, cells were harvested and plated at a concentration of 2 million/mL and stimulated with estradiol and/or CpG (TLR9 ligand) or PBS control for 18 hours. Frequency of PDC-TREM+ BM derived pDCs obtained from WT NZM (n=3) mice after treatment with 1ug/mL of CpG and increasing amount of estradiol (1-10nM) **(A)** Frequency of PDC-TREM+ BM derived pDCs obtained from WT (n=5) and ERαKO (n=4) B6 mice after treatment with 10nM of estradiol and low amounts of CpG (0.1 or 0.25ug/mL) **(B)**. Data obtained in 2 separate experiments.

PDC-TREM. The literature shows that estradiol can induce optimal DC activation in the presence of suboptimal levels of TLR ligand. Therefore, we hypothesized estradiol may induce an effect on PDC-TREM when TLR ligand levels are suboptimal. To test this hypothesis we added 10nM of estradiol and 0.1 or 0.25ug/mL of TLR9 ligand to BM derived pDCs cultures. Under these conditions we did not detect any change in the frequency of pDCs expressing PDC-TREM with the addition of estradiol (Figure 34B). However, we detected a slight reduction in the frequency of PDC-TREM+ pDCs with the addition of estradiol to BMDC cultures from ERαKO B6 mice (Figure 34B). This finding, although preliminary, suggests the ERαKO protein may act as a negative regulator of TLR ligand induced PDC-TREM expression. This hypothesis is in support of the finding that the ERαKO mice are protected from disease, however, the full ERα deficient mice are not (Cunningham, manuscript in preparation). This observation suggests the truncated ERαKO protein may be protective in disease. Therefore a future study examining this dominant negative mechanism may be useful. Additionally, the summation of these experiments suggests that estradiol/ERα "preprogramming" of the

pDCs may play a role in PDC-TREM expression. The pre programming effect of estradiol/ERα on pDCs IFNα production has been shown in previous studies[16].

Exposure of BM derived pDCs to IFNα during Flt3L culture enhances the reduction of PDC-TREM expression on ERαKO pDC.

We sorted pDCs from Flt3L ligand driven BM cultures from B6 mice and detected a reduction in TLR mediated PDC-TREM expression on ERαKO pDCs. We also stimulated whole Flt3L driven BM cultures from WT and ERαKO NZM mice with TLR9 ligand and detected a significant and repeatable reduction in the frequency of PDC-TREM+ pDCs in cultures from ERαKO NZM mice. We then stimulated whole Flt3L driven BM cultures from WT and ERαKO B6 mice with TLR9 ligand and did not detect a significant decrease in PDC-TREM expression on ERαKO B6 pDCs.

Figure 35

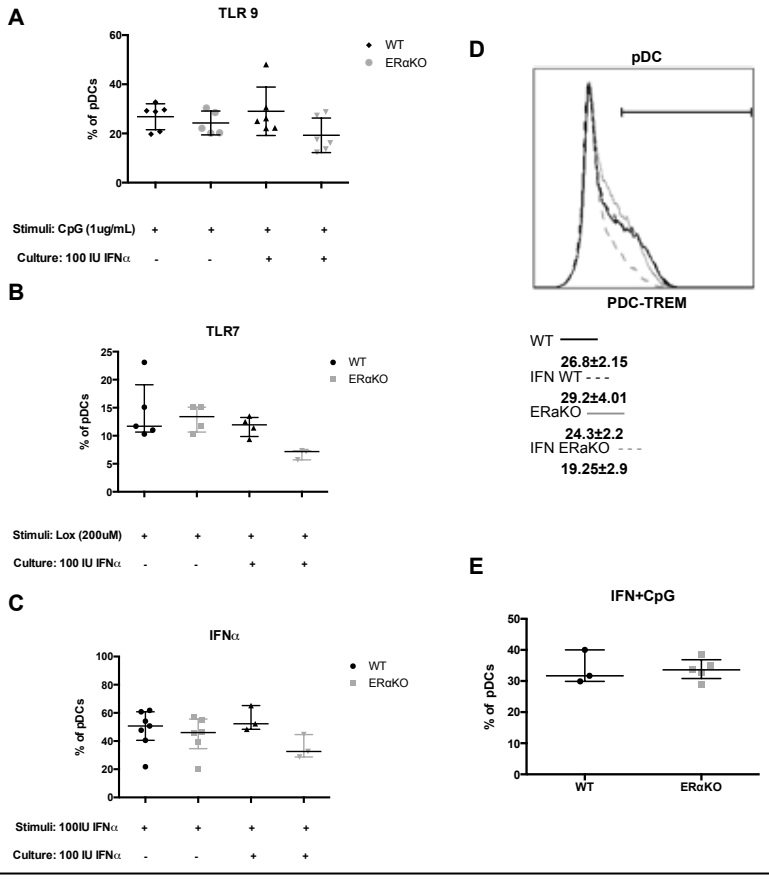


Figure 35. TLR ligand induced PDC-TREM expression on BM derived DCs pre-treated with IFNα. BM from WT (n≥4) and ERαKO (n≥4) female B6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions with or without 100IU of recombinant mouse IFNα. On day 7 cells were harvested and the entire mixture of pDCs and cDCs were plated at a concentration of 2 million/mL and stimulated with 1ug of CpG (TLR9 ligand) **(A)**, 200ug/mL of loroxibine (TLR7 ligand) **(B),** or 100IU of IFNα **(C)** for 18 hours. The DCs were alsot reated with 1ug/mL of CpG and 100IU of IFNα simultaneously **(E).** Frequency of PDC-TREM$^+$ pDCs (CD11c$^+$B220$^+$) was measured with flow cytometry**.** Representative flow cytometry, histogram of TLR9 stimulated BMDCs **(D).** Results are expressed as the median ±SD. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

A major difference between the NZM and B6 Flt3L cultures is the presence of an inflammatory state in the NZM mice, both *in vitro* in the culture dish and prior to culture in the mouse. Whether the inflammatory state is actively present in the culture dish or the precursor cells have been exposed to an inflammatory environment *in vivo*, in this state of inflammation pDCs may become more sensitive to TLR stimulation. Increased sensitivity to TLR 9 ligand may explain the difference in PDC-TREM expression between NZM and B6 mice. Furthermore, we saw a reduction in TLR mediated PDC-TREM expression on sorted pDCs from B6 mice. Since the sorted pDCs make up the entirety of the culture, the cells will have a greater availability of TLR 9 ligand compared to mixed cultures. Thus, explaining the difference between sorted and mixed pDCs cultures in TLR mediated expression of PDC-TREM. Based on this evidence we hypothesized an inflammatory state enhances the reduction in TLR mediated PDC-TREM expression on ERαKO pDCs. To test this hypothesis we treated Flt3L driven BM cultures with 100U/mL of IFNα on day 0 of culture and measured TLR and IFNα mediated PDC-TREM expression on pDCs at day 7. We found that TLR 9 ligand, TLR 7 ligand, and IFNα treatment of BM derived DCs treated with IFNα showed trends towards reduced PDC-TREM expression on pDCs from ERαKO mice compared the pDCs from ERαKO mice that were not treated with IFNα during culture (Figure 35). This finding suggests that the reduction in ERαKO pDC PDC-TREM expression is mediated by both TLR ligands and IFNα and that both of these signals are required for ERα's impact on PDC-TREM expression. To determine if the effect was because of pre-programming provided by the inflammatory environment or was an immediate effect of IFNα signaling we treated BM derived DC cultures with both TLR 9 ligand and IFNα for 18 hours. Under these conditions, there was no change in

PDC-TREM expression between WT and ERαKO pDCs, indicating that IFNα's impacts PDC-TREM expression by 'programming' the pDCs for sensitivity to future TLR stimulation. Since the change in PDC-TREM expression in the IFNα pre-treated cultures was the results of reduced expression of PDC-TREM on ERαKO pDCs rather than an increase of PDC-TREM on WT pDCs, these findings also suggest the ERαKO protein may act as a negative regulator of pDC activation. Given the findings in with estradiol treatment and disease protection provided by the ERαKO, this dominant negative effect should be studied in greater detail in the future.

ERα signaling positively regulates pdc-trem mRNA levels prior to TLR stimulation

To investigate the mechanism by which the functional mutation of ERα reduced TLR mediated PDC-TREM we hypothesized ERα could impact three aspects of pDC development that contribute to pDC functionality. The literature demonstrates estradiol/ERα impacts pDC type I IFN production by 'pre-programming' the pDCs *in vivo* to be more responsive to *ex vivo* TLR stimulation. Although some literature supports a stimulatory role of estradiol at the time of TLR stimulation, our data suggests ERα impacts PDC-TREM expression by pre-programming pDC function during development. We investigated the impact of ERα on pDC expression of the pDC master regulator gene E2-2, TLRs7 and 9, and PDC-TREM.

E2-2 is the master transcriptional regulator of pDC development. Without E2-2 pDCs cannot develop to maturity or produce type I IFN [71]. Furthermore, if E2-2 expression is lost in mature pDCs the cells will revert back to a conventional DC phenotype [72]. We hypothesized ERα may impact the expression of E2-2 and thereby t

Figure 36

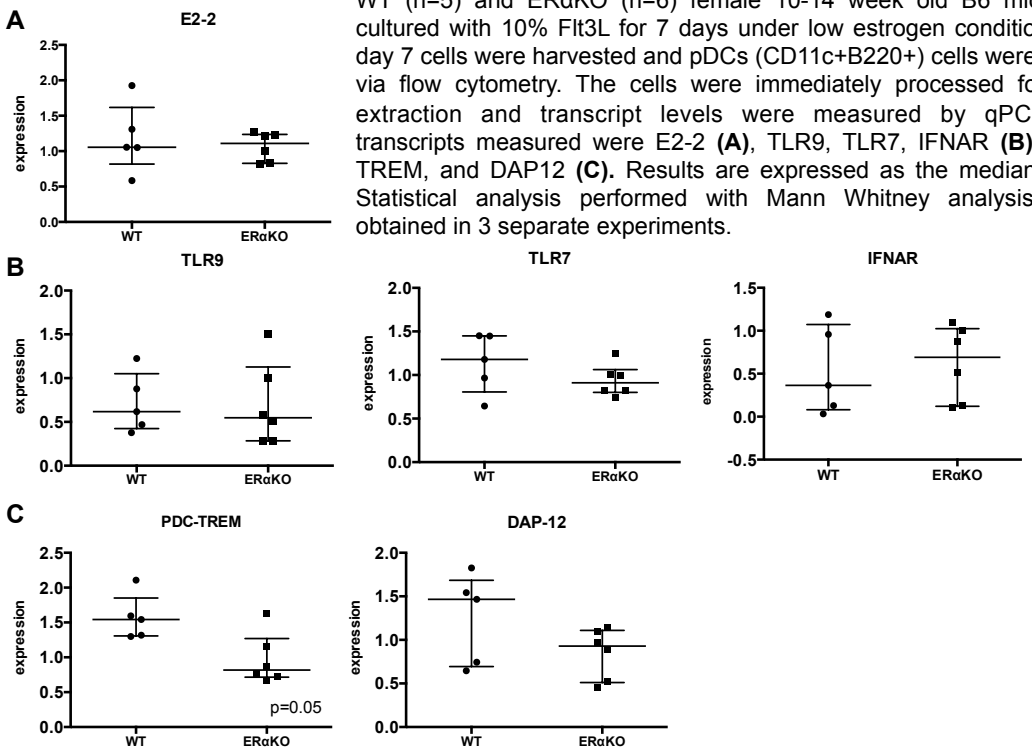


Figure 36. Expression of pDC genes in TLR naïve pDC. BM from WT (n=5) and ERαKO (n=6) female 10-14 week old B6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. On day 7 cells were harvested and pDCs (CD11c+B220+) cells were sorted via flow cytometry. The cells were immediately processed for RNA extraction and transcript levels were measured by qPCR The transcripts measured were E2-2 **(A)**, TLR9, TLR7, IFNAR **(B),** PDC-TREM, and DAP12 **(C).** Results are expressed as the median ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

he overall phenotype of the pDCs. To test this hypothesis we measured levels of E2-2 transcripts in Flt3L derived pDCs from WT and ERαKO B6 mice. E2-2 transcript levels were equal in WT and ERαKO pDCs (Figure 36 A). This finding suggests ERαKO pDCs develop into fully mature pDCs. This finding also establishes that ERαKO, although important in pDC development, does not impact the overall pDC phenotype of cells produced by the Flt3L culture.

We hypothesized ERαKO pDCs are less responsive to TLR ligands because they express reduced levels of TLR 7/9 and IFNα receptor (IFNAR). To test this hypothesis we measured the levels of the transcripts for these receptors in BM derived pDCs from WT and ERαKO B6 mice. There was no difference in transcript levels of TLR7, TLR9, or IFNAR detected in between WT and ERαKO pDCs (Figure 36B). Since reliable

Figure 37

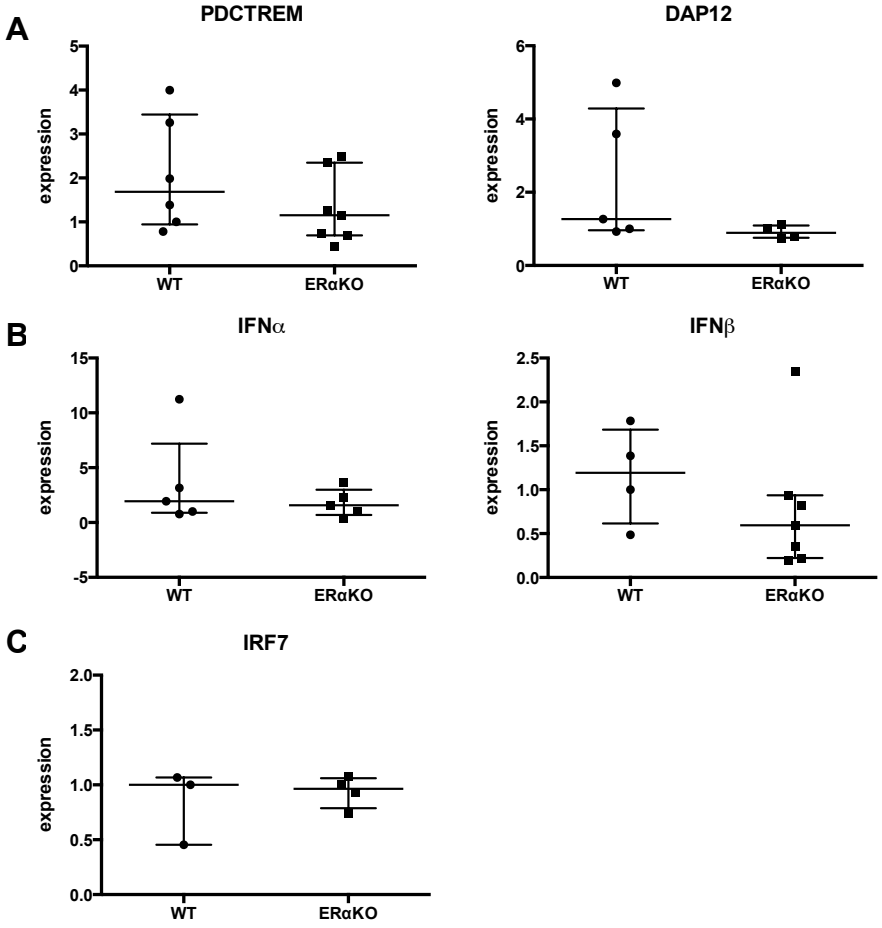


Figure 37. Expression of pDC genes after TLR stimulation. BM from WT (n=6) and ERαKO (n=6) female 10-14 week old B6 mice was cultured with 10% Flt3L for 7 days under low estrogen conditions. On day 7 cells were harvested and pDCs (CD11c+B220+) cells were sorted via flow cytometry. The sortd cells were plated at a concentration of 5x105/mL and stimulated with TLR9 ligand (CpG 1ug/mL) for 5 hours. The cells were immediately processed for RNA extraction and transcript levels were measured by qPCR The transcripts measured were PDC-TREM, DAP-12 **(A)**, IFNα and IFNβ **(B),** and IRF 7 **(C).** Results are expressed as the media ±IQR. Statistical analysis performed with Mann Whitney analysis. Data obtained in 3 separate experiments.

antibodies for TLR7 and 9 are not available this finding provides the best evidence that ERα does not impact TLR7/9 expression in pDCs.

Since PDC-TREM expression was reduced in ERαKO pDCs compared to WT pDCs after TLR stimulation we hypothesized the ERαKO pDCs have less transcripts of PDC-TREM and PDC-TREM associated molecules. To test this hypothesis we measured the transcripts of PDC-TREM and its associated signaling molecule DAP12 in BM derived pDCs from WT and ERαKO B6 mice prior to stimulation with TLR ligands. The ERαKO pDCs expressed lower levels of PDC-TREM mRNA compared to WT pDCs (Figure 36C). Additionally, there was a trend of reduced DAP12 transcripts in the ERαKO pDCs compared to PDC-TREM (Figure 36C). The reason for DAP12 and PDC-TREM levels being reduced at different rates could be that DAP-12 acts a signaling molecule for other pDCs surface receptors. Although PDC-TREM surface expression is only detected after TLR stimulation, PDC-TREM mRNA is detected in unstimulated pDCs [84]. Prior to TLR stimulation ERα signaling positively impacts PDC-TREM mRNA levels and may increase the expression of he adaptor molecule DAP12. The regulation of PDC-TREM mRNA may represent the mechanism by which ERα signaling pre-programs pDCs to express increased levels of surface PDC-TREM after TLR stimulation.

ERα signaling does not regulate pdc-trem mRNA levels after TLR stimulation

To determine if ERα signaling also reduced PDC-TREM mRNA levels after TLR stimulation we measured the expression of PDC-TREM and DAP-12 in pDCs from B6 WT and ERαKO mice 5 hours after TLR stimulation. After TLR stimulation ERαKO pDCs did not express significantly lower levels of PDC-TREM or DAP-12 transcripts (Figure 37A) This finding suggests that ERα signaling does not likely impact PDC-TREM and DAP-12 expression after TLR stimulation. We also measured express of

IFNα and IFNβ transcript levels are the 5 hours time point post TLR stimulation. There was no change in IFNα and IFNβ transcript levels between genotypes (Figure 37B). Although, this finding suggest ERα signaling does not impact IFN production, the lack of difference is likely due to the time point selected. These finding suggest ERα signaling does not regulate PDC-TREM expression after TLR sitmulation, but rather through an effect of naïve pDC expression of PDC-TREM transcripts prior to TLR stimulation.

Estrogen response elements in PDCTREM promoter

To determine if the PDC-TREM promoter contains estrogen receptors binding sites, estrogen response elements (EREs), we used matinspector software to identify EREs in the promoter of PDC-TREM. We searched for EREs located within 5,000 BPs upstream of the PDC-TREM exon 1. The software identified 6 EREs within the PDC-TREM promoter. Two of these EREs were specific for ERβ. The remaining 4 were specific for ERα. The ERα EREs were located at position 1082, 2867, 4065, and 4706 (with 5000 being exon1) (Figure 38A). The ERE located at the position 1082 contained the ERE core sequence GACC (Figure 38B and C). The three remaining EREs contained the GTCA core sequence (Figure 38D and E). The EREs all contained core sequences as well as >85% similarity with the surrounding 19bp around the core sequences (Table 1). Based on this data, it is possible ERα binds directly to the PDC-TREM promoter.

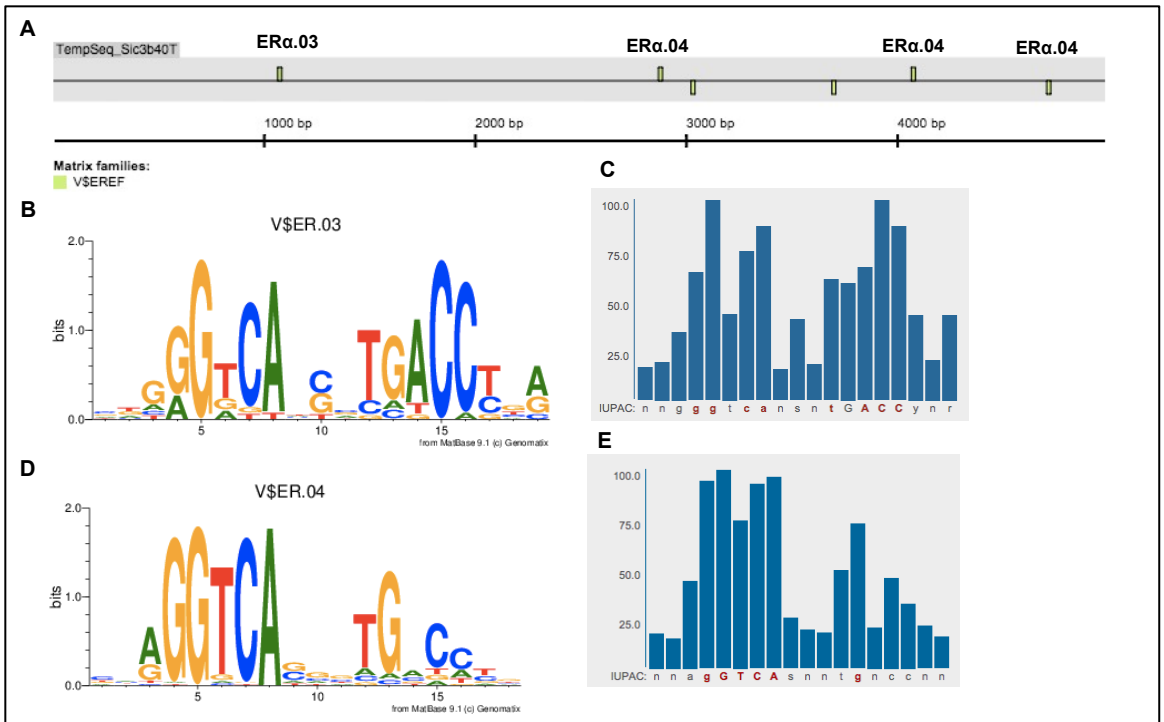


Figure 38. Potential estrogen response elements located in the PDC-TREM promoter. We identified 6 estrogen response elements (EREs) in the 5,000bp upsteam of PDC-TREM exon 1 **(A)**. Matinspector software was used to obtain potential binding regions with 100% core sequence similairty and .85% matrix similarity. The matrix for ERα.03 is depicted in **(B and C)** and the matrix for ERα.04 is depicted in **(D and E).** In C and E nucleotides in capital letters denote core sequence and nucleotides in red represent highly conserved (60%) content.

Table 1. Estrogen Response Elements in PDC-TREM Promoter

Family	Matrix	Start position	End position	Strand	Core sim.	Matrix sim.	Sequence
ERE	ERα.03	1064	1082	+	1	0.826	aagaggcaatgaGACCtag
ERE	ERα.04	2867	2885	+	1	0.912	ggggGTCAggctgggctca
ERE	ERβ.01	3021	3039	-	1	0.923	acagGTCAaatggccttgt
ERE	ERβ.01	3688	3706	-	1	0.91	gcagGTCAccctgggagga
ERE	ERα.04	4065	4083	+	1	0.902	attgGTCAgtctgccggca
ERE	ERα.04	4706	4724	-	1	0.874	cctgGTCAgggtgggggat

Summary

- ERα signaling positively impacts TLR mediated PDC-TREM surface expression on BM derived pDCs from NZM and B6 mice
- Estradiol treatment at time of TLR stimulation does not impact PDC-TREM surface expression in WT BM derived pDCs.
- WT and ERαKO BM derived naïve pDCs from B6 mice express equal levels of E2-2, TLR7, TLR9, and IFNAR transcripts.
- ERαKO BM derived naïve pDCs from B6 mice have significantly less PDC-TREM transcripts and a trend towards reduced DAP-12 transcript levels compared to WT BM derived naïve pDCs
- Estradiol treatment and pre-treatment with IFNα reduced TLR mediated PDC-TREM surface expression on BM derived pDCs from B6 ERαKO mice.

4.4 Discussion

PDC-TREM protein is expressed on the surface of pDCs after TLR stimulation and is not expressed on TLR ligand naïve pDCs [84]. However, in naïve pDCs PDC-TREM transcripts are detectable [84]. We found that ERα signaling positively impacts surface expression of PDC-TREM after TLR stimulation. Additionally, ERα signaling positively impacts PDC-TREM transcript levels in TLR ligand naïve pDCs. These findings suggest ERα signaling impacts TLR mediated PDC-TREM expression by mediating naïve pDC's potential to respond to TLR ligands. ERα's impact on PDC-TREM transcripts prior to TLR stimulation likely accounts for the majority of regulation because estradiol treatment at the time of TLR stimulation does not alter PDC-TREM surface expression. Therefore, ERα signaling likely 'pre-programs' a pDC's ability to respond to TLR ligand. The pre-programming by estradiol/ERα has been previously reported. Seillet et al found *in vivo* estradiol signaling through ERα increased *in vitro* TLR mediated pDC IFNα production [16]. Our results suggest that PDC-TREM expression is likely also regulated by estradiol mediated pre-programming. Additionally, our finding provides a possible mechanism by which ERα signaling impacts IFN production.

Inflammation likely impacts ERα's role in PDC-TREM expression. We reported enhanced differences in pDC activation between WT and ERαKO pDCs from NZM mice compared to B6 mice both *in vivo* and *in vitro*. In vivo, it is tempting to attribute this enhancement to the presence of additional immune stimuli in NZM mice. However, when pDCs are provided equal doses of TLR ligand *in vitro*, pDCs from WT and ERαKO NZM mice maintain the greater differences in pDC activation compared to B6 mice. This

finding suggests a mechanism beyond increased stimuli levels is responsible for the difference. We hypothesized the increased inflammation in NZM mice contributes to ERα's impact on TLR mediated pDC activation. To test the hypothesis we used IFNα to simulate an inflammatory environment in the culture during Flt3L mediated DC development. We found that the addition of IFNα during culture broadened differences in PDC-TREM expression between TLR stimulated WT and ERαKO pDC from B6 mice. Although this finding was not significant for each individual stimuli, the trend was seen in each stimuli. This finding suggests that inflammation enhances ERα's role in TLR mediated pDC activation. In the literature, evidence shows that ERα signaling impacts inflammatory responses [56]. Although controversy exists in this field, ERα likely enhances and represses inflammation depending on the scenario. Precisely how ERα takes on this dual role is under investigation.

Inflammation also regulates ERα signaling. In an *in vivo* mouse model of joint inflammation, affected joints had reduced levels of ERα protein compared to unaffected joints [107]. Molecularly, in a breast cancer cell line NFkB repressed ERα and β signaling activity and RelB via Blimp1 inhibited the synthesis of ERα [108]. Additionally, NFkB and ERα can act synergistically to regulate gene expression [108]. IFNα also impacts ERα signaling. Treatment of a breast cancer cell line with IFNα increased levels of ER protein [109]. IFNα treatment of mouse spleen cells increased levels of ERα transcripts and protein [110]. Since ERα singling enhances IFN production, this finding suggests there is a positive feedback loop between ERα and IFNα signaling [110].

There is also evidence in DCs for inflammation altering ERα signaling. The requirement for the AF-1 domain of ERα changes depending on if the DCs were grown under inflammatory versus steady state conditions [56]. Also, our laboratory has preliminary data suggesting inflammatory DCs express higher levels of ERα transcripts compared to steady state DCs. Given this evidence, it is possible IFNα enhances the pDC's ability to respond to inflammatory stimuli and our data suggests IFNα enhances the pDC's ability to expression PDC-TREM after TLR stimulation.

The ERαKO mouse is not a model of complete ERα deletion. Rather, it expresses low levels of a mutant ERα protein that lacks the AF1 domain, but maintains the DNA binding, ligand binding, and AF2 domain. This mutant protein resembles both the naturally expressed ERα 46 spice variant and the engineered AF1 mutant (Diagram 3). Our data suggests the mutant ERαKO protein may impact pDC activation. When DCs were pre-treated with IFNα, the difference in PDC-TREM expression increase between genotypes. The increase was a result of ERαKO pDCs expressing reduced levels of PDC-TREM in response to IFNα pre-treatment. Additionally, estradiol treatment did not impact WT pDC expression of PDC-TREM, but it did reduce PDC-TREM expression on ERαKO pDCs. This evidence suggests ERαKO mutant protein may be reducing pDC activation status. Alternatively, the ERαKO pDCs could be less responsive to TLR stimulation because of the hormonal milieu in the mice. The ERαKO mice have significantly higher estrogen and testosterone levels compared to WT mice [1]. Increases in these hormones could provide an anti inflammatory effect. Therefore this possibility should be investigated. However, our laboratory has found that these changes in hormone levels are not responsible for disease protection in the ERαKO mice. When the hormone

levels were normalized in ERαKO NZM mice, the mice were protected from disease. Additionally, the mice were not protected in the absence of estrogen, suggesting ERαKO mutant protein requires estrogen for its function. Although the possibility that estrogen signals through another receptor to protect from disease exists, our laboratory found that the complete absence of ERα does not protect mice from disease. This evidence suggests the presence of estrogen mediated ERαKO mutant signaling is necessary for disease protection in NZM mice. Therefore, it is possible that the ERαKO protein is mediating the reduction in PDC-TREM level.

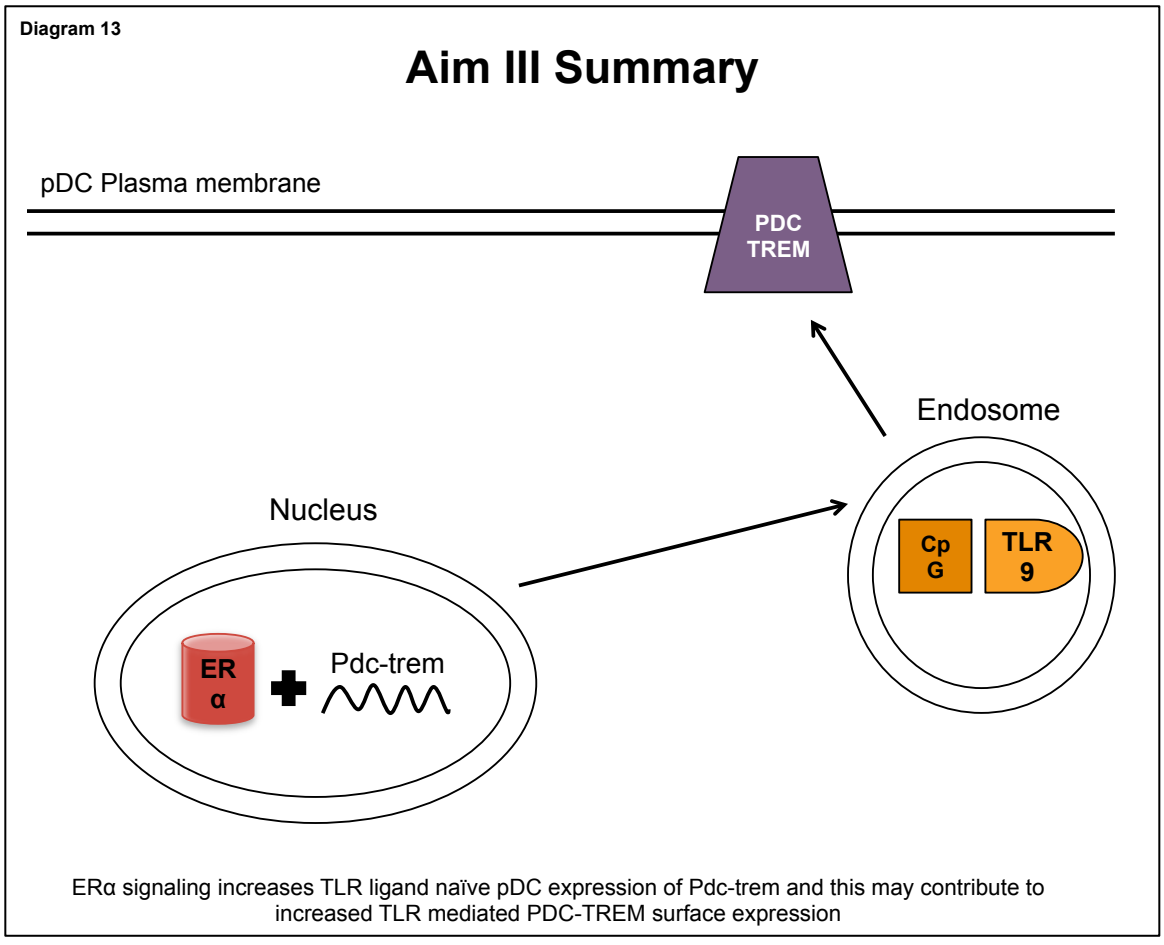
Although, the presence of the natural isoform ERα46 is controversial, there is evidence supporting its existence and function. ERα46 is expressed in human endothelial cells and is located in the plasma membrane, cytosol, and nucleus. The plasma membrane ERα46 mediates rapid changes in eNOS activity [111]. Furthermore, ERα46 can function as a dominant negative to inhibit the transcriptional activities of ERα66 (classical ERα) [112]. There is also evidence the 46KD isoform is expressed and has function in immune cells. In human macrophages, the primary variant of ERα is the estradiol sensitive 46KD isoform. Furthermore its levels increase with the monocyte to macrophage transition [57]. The expression of this splice variant is mediated by a different promoter than the ERα66.

In dendritic cells the engineered ERα AF1 mutant suggests the mutant ERαKO protein may play a role in cell function. Like the AF1 mutant, the ERαKO mutant lacks the AF1 domain. Using the mutant AF1 null ERα, the later stages of inflammatory dendritic cells development was not impacted by a lack of AF1 domain. However, the complete absence of ERα did impact later stages of DC development. This finding suggests the ERα protein lacking AF1 has a function in later stages of DC development.

Since the ERαKO protein resembles the AF1 null mutant, it may also impact late stage DC development. All together this evidence suggests the ERαKO mutant protein may play a role in disease protection. However, we need to strengthen our evidence for the role of this protein in disease.

4.5 Conclusions

ERα signaling positively impacts TLR mediated surface expression of PDC-TREM. ERα signaling likely mediates this effect by pre-programming pDCs to be more responsive to TLR stimulation through a developmental mechanism. (Scheme7). One mechanism that could mediate this effect is by ERα signaling increasing levels of PDC-TREM transcripts in TLR naïve pDCs. The increase in PDC-TREM transcripts occurs without altered expression of TLR7, 9 or IFNAR. This finding suggests ERα signaling impacts signaling factors downstream of TLRs to alter TLR responsiveness in a developmental manner. It is possible the ERαKO mutant protein acts as a dominant negative to cause this effect.



4.6 Future directions

Future work may focus on the regulation of PDC-TREM. Independent of ERα, little is know about the regulation of PDC-TREM. Since transcripts are expressed prior to TLR stimulation, it should be determined if TLR signaling promotes the translation or intracellular trafficking of PDC-TREM. Once this is understood, the effect of ERα on this process can be investigated. Since ERα signaling impacts PDC-TREM transcript levels, it is important to understand if ERα impacts the transcription of the gene or stability of the transcript. If ERα impacts transcription, we should determine if ERα directly regulates the promoter or is it acts indirectly through a different transcription factor, which then regulates gene expression.

Additionally, the possibility of mutant ERαKO protein acting as a dominant negative should be investigated. This may be done by studying PDC-TREM levels in ERα null mice and hormone controlled ERαKO mice. Additionally, work needs to be done to detect this mutant protein in pDCs.

Lastly, ERα signaling impacts a vast amount of cell functions. Therefore, this work should be put into the context of the broader investigation into ERα regulation of immune cell function. Currently, our laboratory is exploring how ERα singling impacts the transcriptome of dendritic cells. This work will likely yield important information into the major cellular processes altered by ERα signaling.

Chapter 5 Closing remarks

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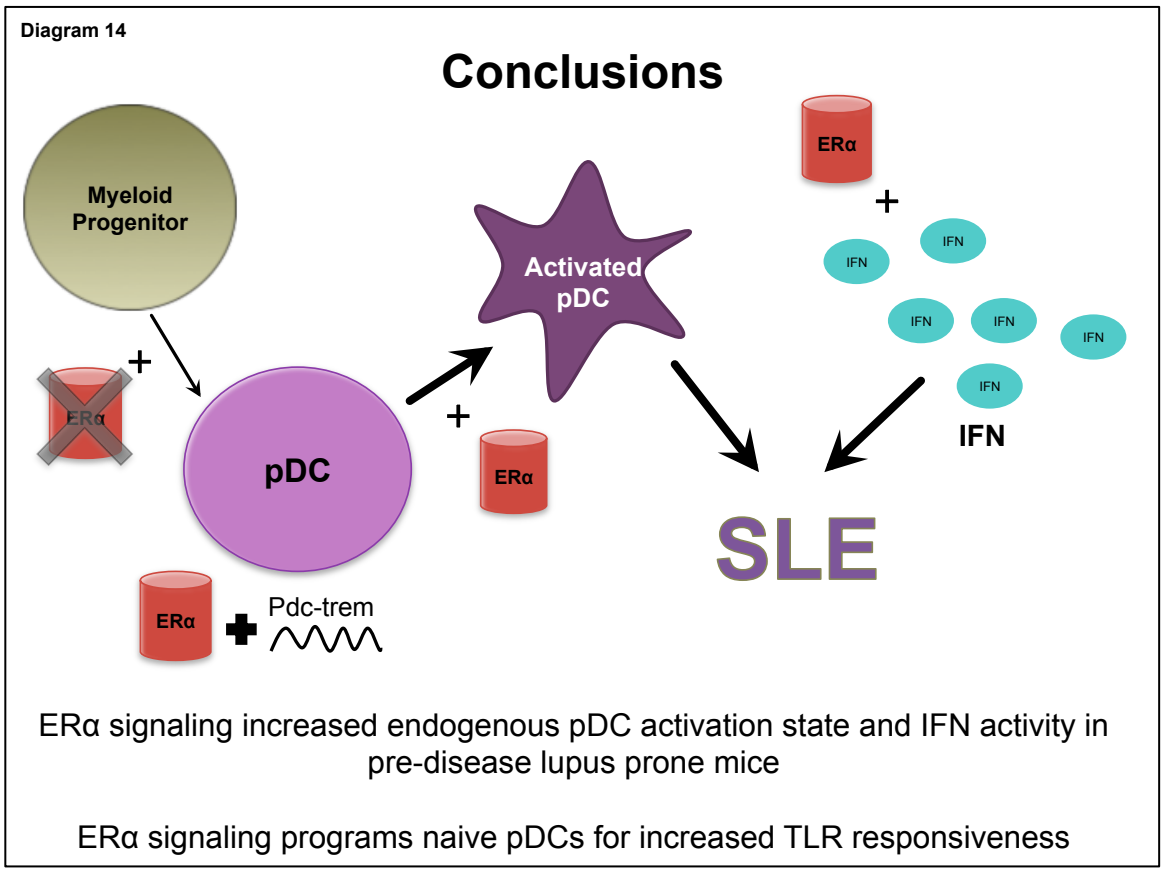

NZM2410 mice lacking functional ERα signaling are protected from disease. The mechanism of disease protection is unknown. In this work we have determined that ERα signaling altered the activation status of pDCs in NZM2410 mice prior to the development of clinical disease without altering pDC number or maturation status. Specifically, ERα signaling positively impacted PDC-TREM and MCHII expression in pre-disease mice. The frequency of PDC-TREM^{+} pDCs is also increased in pre-clinical disease NZM2410 mice compared to controls, suggesting that PDC-TREM expression impacts disease pathogenesis. Simultaneously, ERα signaling positively regulated type I IFN signaling in pre-disease NZM2410 mice. Since PDC-TREM expression is required

for optimal type I IFN production by pDCs, ERα may regulate type I IFN activity via PDC-TREM. Additionally, ERα's impact on PDC-TREM expression and type I IFN signaling may explain disease protection in NZM2410 mice lacking functional ERα signaling.

Since ERα may impact disease by altering pDC expression of PDC-TREM, we explored the mechanism by which this regulation of pDC activation occurs. We found that ERα impacts TLR mediated induction of surface PDC-TREM expression in both B6 and NZM2410 mice, indicating this mechanism occurs in both in the disease and non-disease state. Furthermore, ERα signaling impacts PDC-TREM transcript levels prior to TLR stimulation indicating ERα signaling 'pre-programs' pDCs to express more surface PDC-TREM in response to TLR stimulation. Since PDC-TREM is required to type I IFN production, this finding may represent a mechanism by which ERα alters pDC IFN α production in response to TLR stimulation.

It is important to put this work into the context ERα's role in SLE pathogenesis and the female predominance of SLE. Regarding ERα signaling in disease pathogenesis, ERα is a ubiquitously expressed transcription factor, which affects numerous cellular processes. Thus, it is likely that ERα impacts disease through multiple mechanisms including, but not limited to its impact on pDC activation. Additionally, ERα has been shown to alter both pro and anti-inflammatory processes, suggesting ERα may impact disease differently depending on the timing and cellular environment. Since ERα exerts numerous effects on physiologic processes it is difficult to completely understand its function and role in disease. Thus, our work is important because it addresses a single and specific aspect of ERα function. To further address ERα function in disease, we will

need to use cell specific and conditional ERα knockouts and ERα mutant models. Additionally, we need to place ERα's impact on PDC-TREM transcript levels within the context of ERα's impact on the entire transcriptome to fully understand ERα's role in DC 'pre-programming'. Future work will address these issues. Regarding the female predominance of disease, the mechanism studied in this work along with other work in our laboratory can be translated into human studies. The human studies will allow us to explore how ERα contributes to the SLE sex bias.

Chapter 6 Materials and Methods

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Mice: ERα deficient (ERαKO) mice on the C57BL/6 background (kind gift of Dr. Ken Korach, NIEHS, RTP, NC) were backcrossed for 12 generations to NZM2410 mice (Jackson Laboratory, Bar Harbor, ME) and congenic status was verified as previously described [55]. Female mice between 10 and 14 or 22-26 weeks of age were used for these experiments. All mice were maintained at the Ralph H. Johnson VAMC Animal Care Facility (Charleston, SC) using Institutional Animal Care and Use Committee approved protocols #421 and #498 originally approved August 2008 and 2011 respectively.

Spleen DCs: Spleens were harvested from mice and placed on ice in RPMI-media. The spleens were processed through 70um strainers and depleted of red blood cells with red blood cell lysis buffer (144 mM NH_4Cl and 17 mM Tris, pH 7.6). After washing in estrogen-free RPMI-media (charcoal dextrane treated FBS in phenol red-free RPMI, pennicillin/streptomycin, ampicillin, and L-glutamine), the cells were counted and either placed in TRIzol (Life Technologies, Grand Island, NY) for RNA extraction or stained for flow cytometry. For flow cytometry, 4 million cells were washed 2x in PBS and resuspended in LIVE/DEAD Fixable Dead Cell Stain (Life Technologies) at a concentration 50ul/million cells. Cells were washed 2x in staining buffer (0.5% BSA, 0.02% sodium azide in PBS) and treated with anti-CD16/CD32 for 5 minutes. Cells were then stained for surface antigens with the indicated conjugated antibodies in 50ul of staining buffer per million cells for 25minutes. All staining was performed in the dark on ice. The cells were washed 2x in staining buffer and re-suspended in 0.3 mLs of staining

buffer and acquired on the LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). Analysis was preformed using FlowJo software (Treestar Ind, Ashland, OR). All cells analyzed were first identified as both singlets and live to eliminate debris and dead cells, respectively. pDCs were identified as $CD11b^-$, $B220^+$, $SiglecH^+$. Serial gating was used to identify pDCs by identifying $SiglecH^+$ cells in a pre-selected $B220^+$, $CD11b^-$ population (Supplemental figure 2). cDCs were identified as $B220^-$, $SiglecH^-$, $CD11c^+$. Activation markers were measured on these populations of DCs as indicated. Fluorescence minus one controls were used as indicated to select gates.

Bone marrow DCs: Bone marrow was flushed from the femurs and tibias of mice and processed through a 70um strainer and depleted of RBCs in the same manner as the spleen. The cells were either stained for flow cytometry or cultured to obtain bone marrow derived DCs (BMDCs). Total bone marrow was stained for flow cytometry and analyzed in the same manner as the spleen cells.

For BMDC generation total BM was plated at a concentration of 1.5 $x10^6$ cells per mL in estrogen-free RPMI-media with 10% supernatant from a Flt3L producing cell line (a kind gift of Dr. Stephania Gallucci, Temple University). The BMDCS were harvested on day 7, counted, and re-plated at a concentration of 2 $x10^6$ cells per mL in estrogen-free RPMI for TLR ligand treatment. If indicated, pDCs were sorted from the total BMDC population using fluorescent activated cell sorting (FACS) and plated at a concentration of 2 $x10^6$ cells per mL in estrogen-free RPMI. Cells were sorted under sterile conditions using the same staining protocol as the spleen cells. Samples were sorted on the BD MoFlo Astios High Speed Cell Sorter. pDCs were identified as singlets, live, $CD11b^-$,

$CD11c^+$, $B220^+$. The pDCs or total BMDCs were treated with either PBS or CpG DNA (TLR9 agonist) at 1ug/mL. After 18 hours, the cells were scraped from the plates and washed in PBS to prepare for staining for flow cytometry. One million cells were stained in the same manner as the spleen cells. BMDC populations were identified as followed; pDCs: $CD11c^+$. $B220^+$, mDC: $CD11c^+$, $B220^-$, $CD11b^+$, and LDCs: $CD11c^+$, $B220^-$, $CD11b^-$.

Antibodies and reagents: Fluorescent conjugated antibodies including anti-mouse CD11c, CD11b, B220, SiglecH, mPDCA1, PDC-TREM, MHC II (I-A/I-E), and CD40 were purchased from Biolegend (San Diego, CA). PE-conjugated anti-mouse Ly49Q was purchased from MBL International (Woburn, MA). LIVE/DEAD fixable near-IR dead cell staining kit was purchased from Life Technologies (Grand Island, NY). Mouse CpG DNA was purchased from Hycult Biotech (Canton, MA). All ELISAs were purchased from BioLegend (San Diego, CA) expect for the IFNα ELISA that was purchased from PBL assay science (Piscataway Township, NJ).

RNA preparation: RNA was extracted from spleen cells using a TRIzol/RNeasy hybrid protocol as described previously [15]. Briefly, an appropriate amount of TRIzol was added to the cells and incubated at room temperature for 30 minutes. Choloroform (0.2mLs per 1mL of TRIzol) was added and hand shaken for 1 minute. The choloroform/TRIzol solution rested at room temperature for 3 minutes before being spun at 4°C for 15 minutes. The clear supernatant was removed and mixed with an equal volume of cold 70% ethanol. The solution was then added to the Qiagen RNeasy column

for further purification via the manufactures instructions. DNA was removed using on column digestion with the Qiagen RNAse free DNase kit. RNA was eluted in RNAse free water and immediately processed for reverse transcription.

qPCR: 1ug of RNA was reverse transcribed to cDNA using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA). qPCR was preformed on the CFX Connect Real-Time system (Bio-rad) using master mixes containing iQ SYBR Green Supermix (Bio-rad), nuclease free water, primers (10nM) and cDNA. Primers for Mx-1, Cxcl-10, ISG-15 were synthesized by Integrated DNA Technologies (Coralville, Iowa) and the IRF-7 and GAPDH primers were purchased from SA Biosciences (Frederick, MD). The PCR conditions were 3m at 95°C, 40 cycles of 15s at 95°C, 30s at 60°C, 30s at 72°C. Melt curve analysis began at 55°C for 10s and increased every 10s by 0.5°C for 80 cycles. All samples were performed in triplicate, no-cDNA controls were included for each primer, and melt curve analysis was used to determine if the desired product was being detected. Expression levels were calculated by comparison to the housekeeping gene (GAPDH) for each sample with the Bio-Rad CFX manager software.

ISG15 F 5’ CAG AAF CAF ACT CCT TAA TTC 3’

R 5’ AGA-CCT-CAT-ATA-TGT-TGC-TGT-G 3’

Mx-1 F 5’ GAT-CCG-ACT-TCA-CTT-CCA-GAT-GG 3’

R 5’ CAT-CTC-AGT-GGT-AGT-CAA-CCC 3’

Cxcl-10 F 5’ GGA TGG CTG TCC TAG CTC TG

R 5’ TGA GCT AGG GAG GAC AAG GA 3’

Statistics: Statistical analysis was performed with GraphPad Prism software. Normality was assessed. If data was normally distributed, a student's T-test was used. If data was not normally distributed, a Mann-Whitney analysis was used. Data are expressed as mean with error bars representing SD.

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