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Novel mechanism for estrogen receptor alpha modulation of murine lupus

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ABSTRACT

Female sex is a risk factor for lupus. Sex hormones, sex chromosomes and hormone receptors are implicated in the pathogenic pathways in lupus. Estrogen receptor alpha (ERα) knockout (KO) mice are used for defining hormone receptor effects in lupus. Prior studies of ERα KO in lupus have conflicting results, likely due to sex hormone levels, different lupus strains and different ERα KO constructs. Our objective was to compare a complete KO of ERα vs. the original functional KO of ERα (expressing a short ERα) on disease expression and immune phenotype, while controlling sex hormone levels. We studied female lupus prone NZM2410 WT and ERα mutant mice. All mice (n = 44) were ovariectomized (OVX) for hormonal control. Groups of each genotype were estrogen (E2)-repleted after OVX. We found that OVXed NZM mice expressing the truncated ERα (ERα short) had significantly reduced nephritis and prolonged survival compared to both wildtype and the complete ERαKO (ERα null) mice, but surprisingly only if E2-repleted. ERα null mice were not protected regardless of E2 status. We observed significant differences in splenic B cells and dendritic cells and a decrease in cDC2 (CD11b + $CD8^-$) dendritic cells, without a concomitant decrease in cDC1 (CD11b-CD8a+) cells comparing ERα short to ERα null or WT mice. Our data support a protective role for the ERα short protein. ERα short is similar to an endogenously expressed ERα variant (ERα46). Modulating its expression/activity represents a potential approach for treating female-predominant autoimmune diseases.

1. Introduction

Nine out of ten patients diagnosed with systemic lupus erythematosus (SLE) are female, thus biologic sex is key in disease susceptibility. The mechanisms underlying the sex disparity in SLE are multifactorial, and likely involve the sex chromosomes, sex hormones and their receptors. There is an established correlation between estrogen and lupus disease expression that is strongly supported by epidemiologic data. Lupus disease incidence is highest during reproductive years, when women are most hormonally active, in contrast to premenarche and post-menopause when lupus incidence is lower and the female:male ratio much less profound.

Extensive evidence indicates that estrogen (E2), usually via ERα signaling, has significant immunomodulatory effects on most immune cell types, both developmentally and functionally. Testosterone (T2) and other sex hormones also have known immunomodulatory effects [1–5]. In murine lupus, manipulating sex hormones via classic ovariectomy and castration experiments demonstrated clear roles for E2 and T2 in exacerbating or ameliorating lupus disease expression, respectively [6–8]. However, results from studies where ERα was deleted or disrupted were not as consistent. For example, ERα deficiency in wild-type B6/129 mice resulted in a lupus-like glomerulonephritis [9]. In contrast, targeted ERα disruption in NZM2410 and MRL/lpr female mice ameliorated glomerulonephritis, without decreasing autoantibody production, while in NZB/NZW F1 lupus-prone mice ERα deficiency resulted in attenuation of glomerulonephritis and decreased autoantibody production [10,11].

Of import, the ERα deficient mice described in all the above reports were studied with intact gonads, which in the setting of ERα disruption, resulted in high serum levels of E2 and T2 in response to elevated GnRH (hypergonadism). Since androgen receptor and estrogen receptor beta are expressed normally in ERα deficient mice, supraphysiologic T2 and E2 represent significant confounders in interpreting these earlier knockout experiments. Recently, we demonstrated that female lupus-prone ERα null mice are in fact not protected from lupus disease expression after ovariectomy indicating the high levels of E2 and T2 might be the mechanism for protection in prior studies of ERα deficiency [12].

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Of equal import, although classic ERα endocrine action is absent in the original ERαKO mice [13], there is residual expression of an N-terminally truncated ERα (rather than complete deficiency of ERα) [14]. We refer to this truncated ERα as ERα short. The ERα short is a potential additional confounder in the earlier ERα deficient murine studies done in lupus-prone mice. This short ERα is missing a critical activation domain (AF-1) resulting in, strictly speaking, a functional knockout of ERα for many endocrine endpoints. ERα short mice are infertile and have physiologic deficiencies wherever classic estrogen action via AF-1 is required (ex. reproductive tissues) [13]. However, the ERα protein expressed by these animals retains an intact DNA-binding domain (DBD), cofactor binding domain, ligand binding domain (LBD) and a second activation domain AF-2, likely preserving some non-classical ERα functions [15]. Thus, mice expressing ERα short may express a murine lupus phenotype different from mice that are ERα null. In this study, we compared ERα short mice to ERα null mice, both on the female NZM2410 lupus-prone background. To control for hypergonadism, test groups of mice were ovariectomized (E2-and T2-depleted), with subsequently E2 repletion (via pellet) or non repletion (placebo pellet).

Unexpectedly, ERα short (missing AF-1) was protective against lupus renal disease, while the ERα null was not. Ovariectomy of mice expressing either ERα short or ERα null resulted in no protection against lupus disease development. The protective effect of the ERα short, but not ERα null, however, was restored by estrogen repletion of the OVXed mice. Thus the mice with the greatest protection against lupus nephritis expressed the ERα short with either endogenous estrogen (ovaries intact) or exogenous estrogen via E2 pellets. These findings indicate there is an active role for ERα short in estrogen-replete mice in modulating disease expression; providing protection not afforded by total deficiency of the full-length ERα. The immune-phenotype of the groups is strikingly different, particularly innate immune cells such as dendritic cells (DCs). Improved understanding of nuclear hormone receptor action may enable targeting specific transcriptional activation domains, as opposed to manipulating hormones, to allow for separation of estrogen effects on different tissues (immune cells versus reproductive tissue, for example). Thus, selective estrogen receptor modulators (SERMs) that are AF-2 agonists, or enhancing expression of endogenous ERα46 short, may prove to be a therapeutically viable approach to treating lupus and other autoimmune diseases where estrogen may play a pathogenic role.

2. Materials and methods

2.1. Mice

Mice were maintained at the Ralph H. Johnson VAMC Animal Facility (Charleston, SC). NZM2410 mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). ERαKO mice (ERα short) on the C57BL/6 background were a kind gift of Dr. Ken Korach (NIEHS) and are also available commercially (Stock No. 004744, The Jackson Laboratory). Mice carrying this *Esr1*^{tm1Ksk} allele express a truncated form of ERα (ERα short), the result of alternate splicing of the transcript [14]. In contrast, mice carrying the *Esr1*$^{tm4.2Ksk}$ allele (Stock No. 026176, The Jackson Laboratory) are ERα null, and have no tissue responses to estrogen or estrogen receptor alpha activity [16]. All experimental mice (n = 44) were female and littermates when possible. All mice were ovariectomized (OVX) pre-disease at 4–8 weeks of age (peri-puberty). Two groups subsequently received 0.25 mg, 90-day sustained release 17β-estradiol pellet, implanted sub-dermally x 2 occurrences (180d) to ensure continuous systemic E2 levels in adult mice (Innovative Research of America, Sarasota, FL, USA).

2.2. Serum anti-dsDNA, serum estradiol, and serum testosterone

Serum was collected throughout the experiment and at time of sacrifice. Serum anti-dsDNA was measured by ELISA assay, as previously described [10]. Estradiol levels were assessed *via* ELISA (Calbiotech, San Diego, CA, USA), with an assay sensitivity of 3 pg/ml; precision: 3.1% (intra-assay), 9.9% (inter-assay). Testosterone serum levels were assessed by radioimmunoassay (RIA) at the University of Virginia Center for Research and Reproduction Ligand Assay and Analysis core.

2.3. Urine protein excretion

Mice were housed in metabolic cages for 24 urine hour collection at 2–4 week intervals starting at 10 weeks of age until sacrifice. To prevent bacterial growth, antibiotics (ampicillin 25 μg/mL, gentamicin 50 μg/mL, chloramphenicol 200 μg/mL) were added to the collection tube. After 24 h, urine quantity was determined and samples were frozen at −20° for future analysis via mouse albumin ELISA with known standards.

2.4. Spleen and kidney processing and renal pathology

Spleens were harvested and kept in RPMI on ice during processing. The spleens were processed through 40 μm strainers and depleted of red blood cells with red blood cell lysis buffer (144 mM NH_4Cl and 17 mM Tris, pH 7.6). Spleen cells were washed twice with cold RPMI before being stained for flow cytometry analysis.

One kidney was digested with DNase I (Roche Life Sciences, Indianapolis, Indiana) and collagenase IV (Sigma Aldrich, St. Louis, MO) and PBMCs were isolated using a Percoll gradient (Sigma Aldrich, St. Louis, MO). A second kidney was divided evenly for renal pathology and IHC. Kidney sections were analyzed in a blinded fashion by Dr. Phillip Ruiz (Department of Pathology, University of Miami School of Medicine, Miami, FL) and graded on glomerular hyper-cellularity, segmental mesangial expansion, neutrophils/cell debris, crescent formation, and necrosis. These scores were combined for a total pathology score similar to the Activity Index used in assessing human lupus renal biopsies. Deposition of IgG and complement component C3 was assessed by immunofluorescence after incubating slides with rabbit anti-mouse IgG FITC (Sigma) and sheep anti-mouse C3 FITC (Sigma). IgG and C3 were graded 0–3 for intensity of staining as previously described (17).

2.5. Staining and flow cytometry

Spleen cells were stained with Panel I: F4/80-Brillaint violet 421 (1:100), CD19-PerCP/Cy5.5 (1:100), CD3-Brilliant violet 605 (1:100), and CD49b-PE (1:400) or Panel II: MHCII-APC (1:200), CD11c-Brilliant violet 605 (1:100), CD8a-Brilliant violet 421 (1:100), CD11b-PE (1:400). Cells were incubated with antibodies for 30 min on ice in the dark. All antibodies were purchased from Biolegend (San Diego, CA, USA). Viability was assessed using LIVE/DEAD Fixable Dead Cell stain (Life Technologies, Carlsbad, CA, USA) at a concentration of 50 μl/million cells. Cells were acquired on an LSR Fortessa cell analyzer (BD Biosciences, San Jose, CA, USA) and analysis was performed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA).

2.6. Statistical analysis

Log rank analysis was used to compare trends in animal survival. For all other experiments, depending on whether data was non-parametric or parametric, either Kruskal-Wallis one-way analysis of variance with post-hoc Dunn's multiple comparison test, or ANOVA with post-hoc Dunnett's test were utilized to test for significance when comparing each group to ERαKO (short) NZM +E2. Standard error of the means was reported where applicable. p values ≤ 0.05 were considered significant.

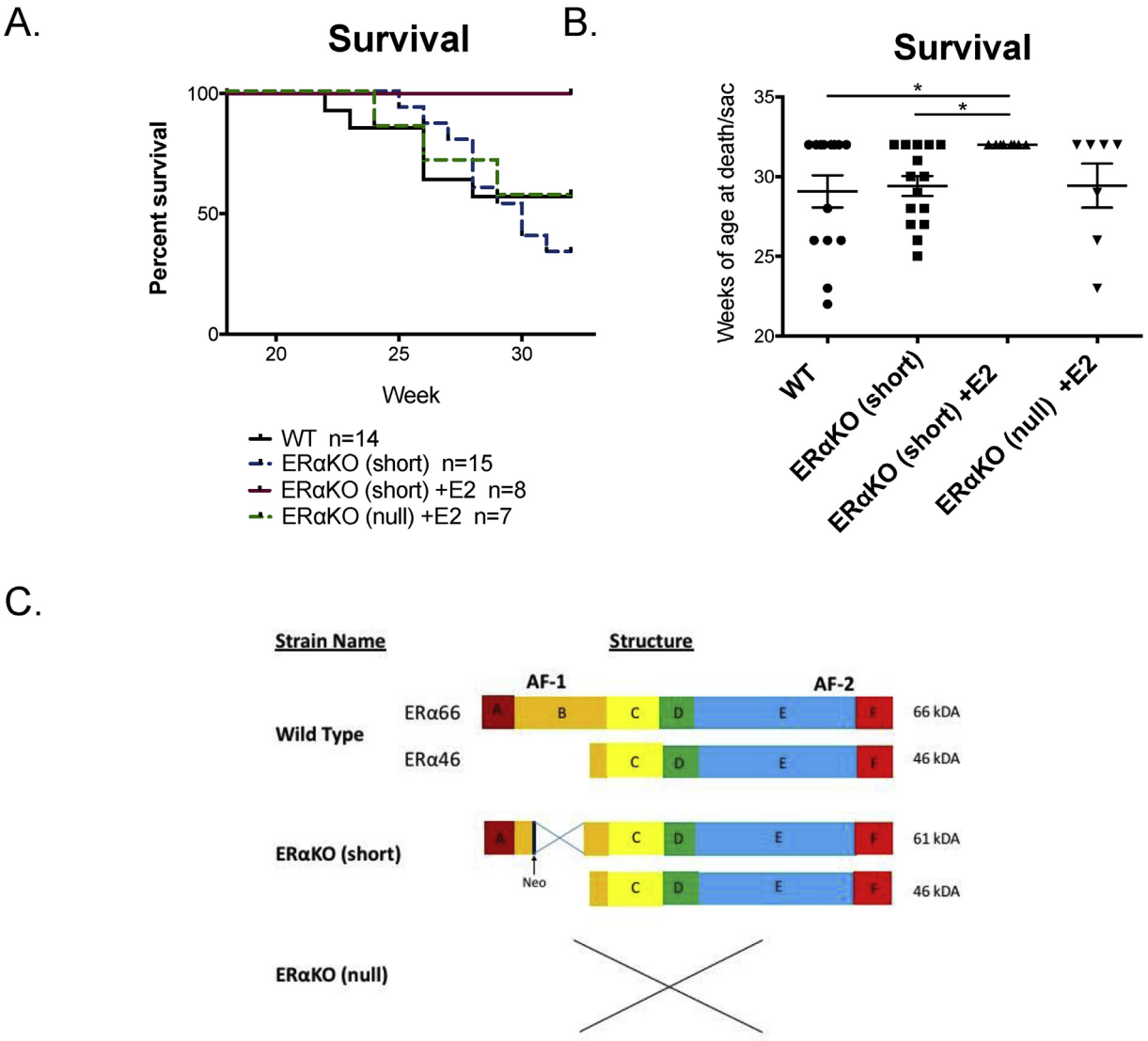

Fig. 1. Survival of ERαKO NZM2410 mice. All mice were female and underwent ovariectomy (OVX). A) Kaplan-Meier curve: 100% of ERα short +E2 mice survived to the 32 week terminal point, whereas survival in other cohorts was 30–57% at 32 weeks. B) Global p-value of differences among all 4 groups (using a log-rank test) was 0.072. Pair-wise comparisons of ERα short +E2 animals to each of the other 3 groups (using a Bonferroni adjusted p-value), resulted in a significantly increased probability of survival in the ERα short +E2 group in comparison with WT and untreated ERα short groups (p = 0.017 and 0.019 respectively), and trended toward significance vs. the ERα null +E2 mice.

3. Results

3.1. Survival effect of functional ERα knockout (ERα short) vs. deletional ERα knockout (ERα null) in murine lupus

All mice were female and were ovariectomized (OVX). Significant differences in mortality were noted between the groups. Despite OVX, WT NZM2410 animals began to die at 23 weeks with 57% alive by 32 weeks (Fig. 1A, black line). This is consistent with known death rates of unmanipulated WT NZM mice previously reported by our group and others [10,17–19]. Thus, early OVX, and resultant estrogen deficiency alone in WT NZM2410 mice, was not sufficient to provide a robust protective effect. In our earlier study, unmanipulated (gonad-intact) ERαKO NZM mice, referred to in this manuscript as ERα short, had significantly improved renal disease and all survived [20]. In this study, when ERα short NZM mice were ovariectomized (T2-and E2-depleted), they were no longer protected from renal disease and died at a similar rate as WT NZM animals (Fig. 1A, blue dashed line). Unexpectedly, however, when the ERα short mice were repleted with estrogen (E2) pellets, disease protection was again present as 100% survived until the pre-determined terminal point of 32 weeks (pink line). This survival is similar to our previous study demonstrating gonad-intact ERα short NZM female mice had a significant survival advantage over WT NZM. These results indicate that E2, but not T2, is required for the protective phenotype observed in ERα short NZM mice.

Consistent with our most recent published study, complete deletion of ERα, referred to as ERα null, was not protective in NZM2410 mice [12]. Survival of OVXed E2-treated ERα null NZM mice at 32 weeks was 57% (Fig. 1A, green line), similar to survival of WT NZM. Thus, OVXed ERα short +E2 mice were the only protected group and disease protection was similar to unmanipulated ERα short mice. We conducted pair-wise comparisons of the ERα short +E2 cohort to the other 3 groups. After Bonferroni adjustment, probability of survival in the ERα short +E2 group was significantly greater than the WT and ERα null groups (p = 0.017 and 0.019 respectively), and trended toward significance vs. the ERα null +E2 mice (Fig. 1A and B). Expressed ERα structures in the individual strains are delineated in Fig. 1C.

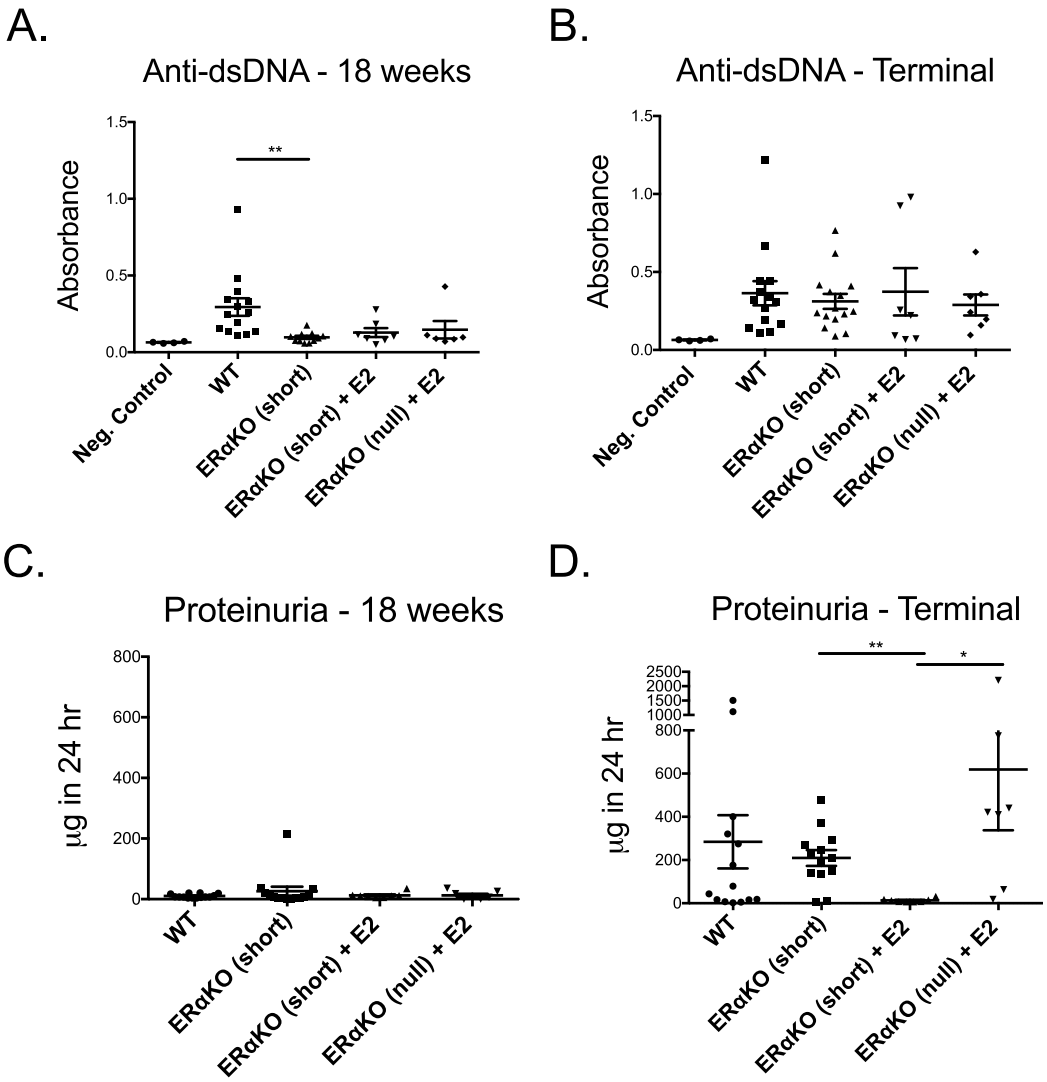

Fig. 2. Anti-double stranded DNA and proteinuria in ERαKO NZM2410 mice. A) Anti-dsDNA levels as measured by ELISA were elevated in WT NZM mice by 18 weeks, and were significantly higher than in ERα short mice. B) By 32 weeks (or at sacrifice), serum anti-dsDNA antibody levels were high in all groups with no significant differences. C) At 18 weeks, no significant proteinuria, as measured by 24 h urine collection on metabolic cages, was noted in any group. D) ERα short +E2 mice maintained low or undetectable levels of proteinuria throughout the study and had significantly less proteinuria compared to untreated ERα short and ERαKO null +E2 mice at the terminal time point.

3.2. Autoimmunity and proteinuria in ERαKO (short) NZM mice vs ERαKO (null) NZM mice

Anti-double stranded DNA (anti-dsDNA) levels were assessed via ELISA at 18 weeks (early stage disease) and at 32 weeks of age (at time of sacrifice). At the early time point, anti-dsDNA levels in WT NZM mice were higher than all other groups, although only significantly increased compared to ERα short mice (Fig. 2A). Since all mice were OVXed, including WT mice, and had near undetectable levels of serum T2 and E2 (Supplemental Fig. 1), the results suggest that lack of sex hormones does not protect against autoantibody development in the NZM2410 model. By 32 weeks, anti-dsDNA levels were elevated in all groups with no significant differences observed (Fig. 2B), suggesting that neither E2 or T2 deficiency, nor differences in ERα expression ultimately impact development of autoantibodies in this model, although they may change the timing of such. This is again consistent with our previous results in unmanipulated ERα short NZM mice in which serum autoantibody levels were elevated, not reduced compared to WT NZM2410 mice, despite significant improvement in other lupus disease parameters.

Urine protein levels (24 h collection) were assessed using albumin ELISA. In general, all groups had low levels of proteinuria at 18 weeks (Fig. 2C). However, by 32 weeks (terminal urine collection), E2-treated ERα short mice had significantly less proteinuria compared to E2-treated ERα null mice (Fig. 2D). This protective effect was not due to differences in T2, since mice had nearly undetectable levels of T2 following OVX. Nor was it due to difference in E2 levels, which were not different between the 2 E2-treated groups (Supplemental Fig. 1). The effect was also not due to differences in levels of ERβ or androgen receptor (AR), since expression of these nuclear hormone receptors is intact in all 4 groups and previously shown to be largely unchanged in ERα deficient mice [21]. These data further support the hypothesis that lupus prone mice expressing ERα short, but not ERα null, are protected from kidney disease, and the effect requires E2.

3.3. Effects of short ERα variant expression on renal histopathology, C3 and IgG deposition in NZM2410 mice

Renal pathology scores, range: 0–18, were determined by a blinded pathologist using H&E stained kidney samples. The most common

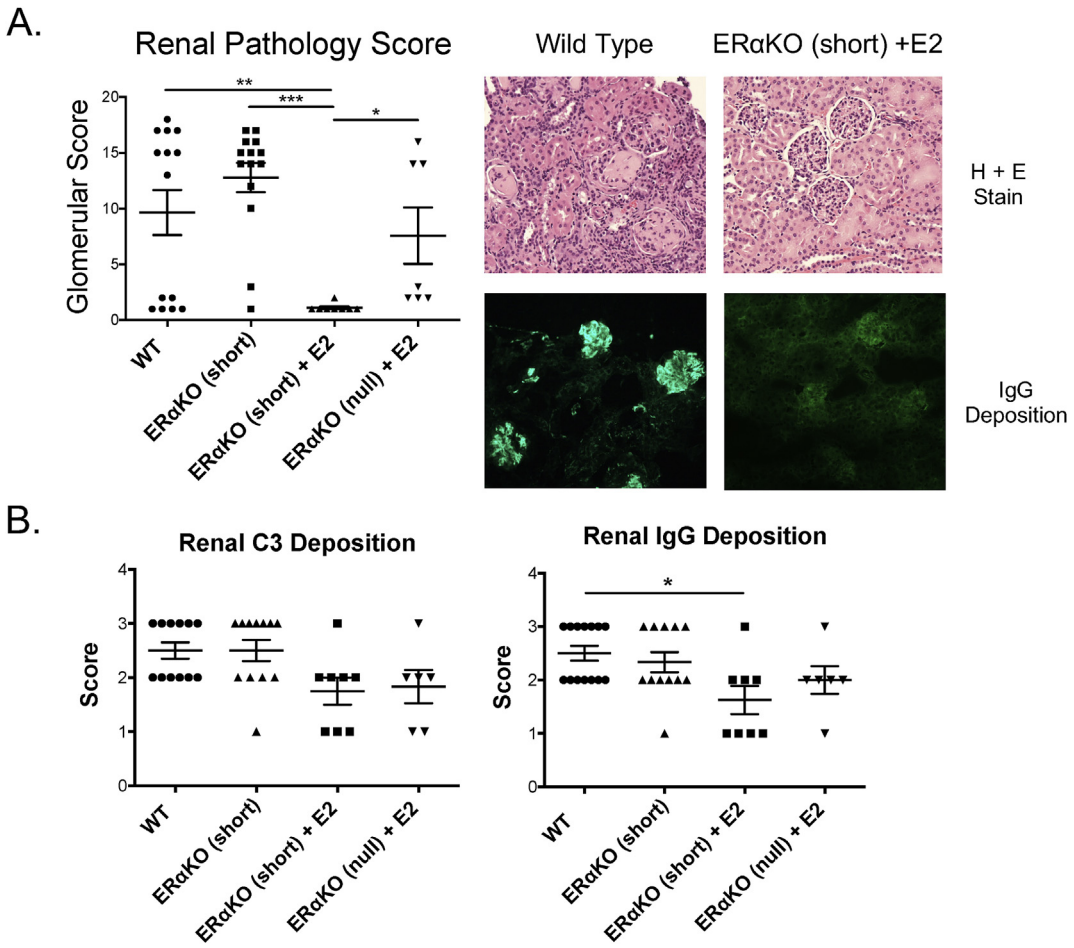

Fig. 3. Renal pathology scores, C3 and IgG Deposition in ERαKO NZM2410 mice. A) Histopathology was scored by a blinded pathologist based on multiple parameters including glomerular hypercellularity, segmental mesangial expansion, membrane thickening, neutrophils/cell debris, crescent formation and focal segmental glomerular sclerosis. ERα short +E2 mice had very low renal pathology scores (< 3), which was significantly lower than the other treatment groups. Other treatment groups were varied, with animals having either high (≥15) or low scores (< 5), indicating few animals with moderate disease. B) C3 and IgG deposition were assessed via immunofluorescence after incubating slides with sheep anti-mouse C3 or rabbit anti-mouse IgG antibodies. Slides were scored on a 0–3 scale by a blinded investigator. WT NZM and untreated ERα short mice had both diseased glomeruli and moderate to high C3 and IgG deposition. E2-treated ERα short and ERα null mice had mild to moderate IgG deposition.

changes contributing to an elevated glomerular score in WT NZM mice were hypercellularity, mesangial expansion, membrane thickening, and focal segmental glomerular sclerosis. Despite early OVX of all mice, WT NZM mice developed diseased glomeruli with moderate-severe IgG deposition, whereas ERα short +E2 mice had normal glomeruli with mild-moderate IgG deposition (Fig. 3A and B). ERα short mice that were OVXed without E2-repletion were not similarly protected, but rather had disease similar to WT animals. ERα null mice developed proliferative renal disease similar to WT NZM (whether receiving E2 or not). These results are thus highly consistent with the proteinuria data; only ERα short +E2 mice were protected. Deposition of C3 and IgG, markers of kidney involvement in lupus disease, was determined by immunofluorescence. Samples were graded on a 0–3 scale by a blinded scorer. While the ERα short +E2 treatment group trended toward lower deposition scores overall, only the IgG deposition was significantly lower compared to WT NZM mice after accounting for multiple comparisons. Both ERα short +E2 and ERα null +E2 groups trended towards lower C3 and IgG scores suggesting that perhaps E2 plays a separate role in immune complex formation/deposition that does not require an intact full-length ERα, similar to the impact on autoantibody production. These data are in agreement with our previous study in which unOVXed ERα short mice had improved renal scores, but no significant reduction in either autoantibody formation or immune complex deposition, suggesting that the local inflammatory responses to immune complex deposition in the kidney are more prominently impacted than other parameters of systemic autoimmunity by ERα.

As in human lupus, the renal scoring system is based on glomerular pathology, but we also assessed chronic inflammation and fibrosis in the tubulointerstitium (TI). NZM mice had concomitant disease in the TI that paralleled the severity of disease in the glomerulus, with reduced severity in the ERα short +E2 mice compared with the other groups (data not shown). We also performed flow cytometry analysis on single cell suspensions prepared from whole kidneys, which provided additional supporting evidence for decreased chronic inflammation in ERα short +E2 mice, which had significantly fewer MHCII+, CD11c+, and CD19 $^{+}$ cells, and trends toward reduced percent of CD3^{+} and CD49b+ cells (supplemental data, Tables S1 and S2). There was also a trend towards reduced percent of cDC2s (CD8a-CD11b+ DCs) from kidneys. cDC1s (CD8a+CD11b-) were exceedingly rare (Supplemental Fig. 4), consistent with previous data in other lupus-prone mice (NZB/W) demonstrating a lack of CD8a+ DCs as a sub-population of renal mononuclear phagocytes [22]. There was no significant change in F4/80 + cells. In summary, only ERα short +E2 mice had significantly lower renal pathology scores (all < 3) and proteinuria and 100% survival rate, as well as reduced local inflammation by flow cytometric analysis of kidney immune cells.

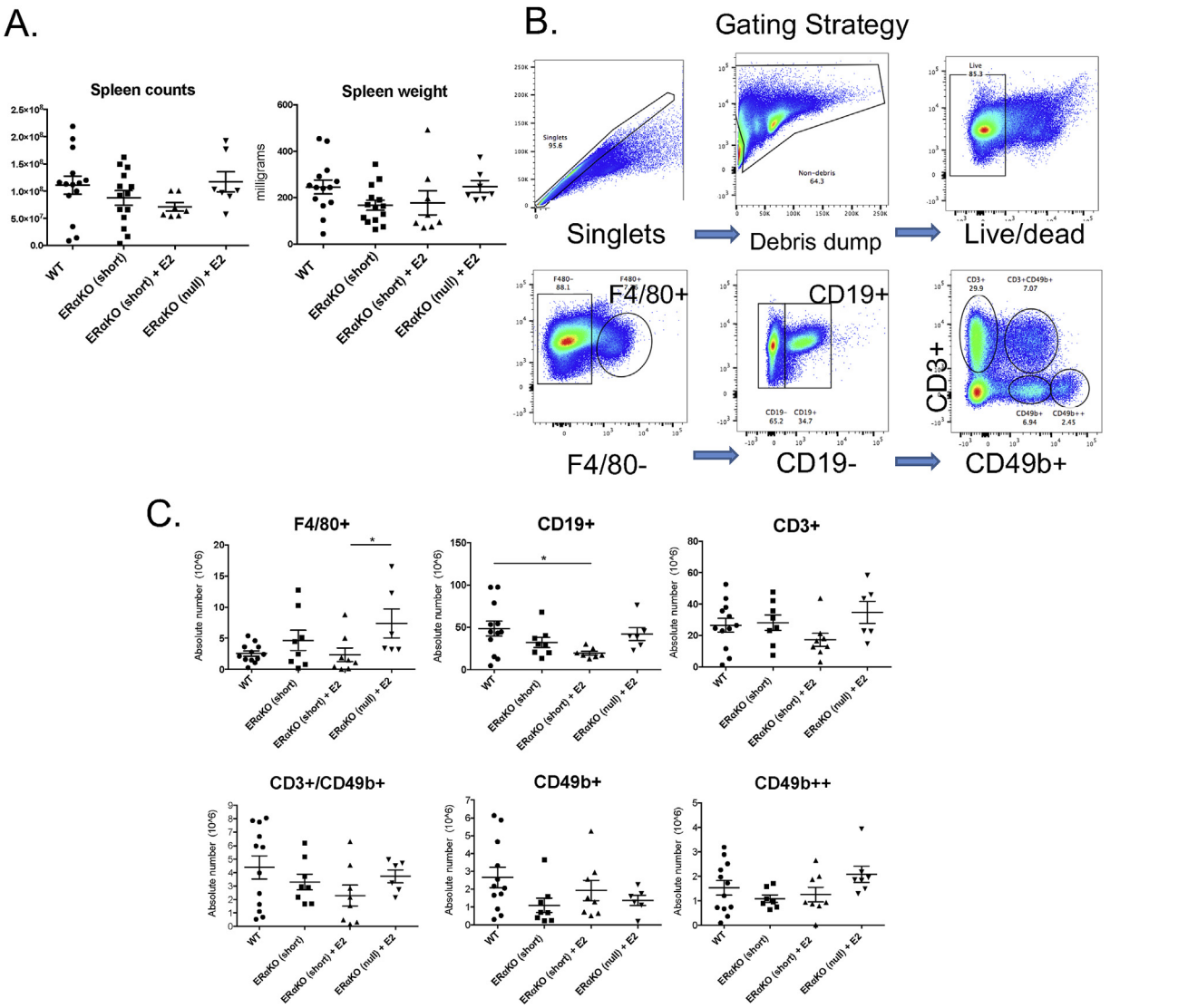

Fig. 4. Spleen cell flow cytometry analysis in ERαKO NZM2410 mice, Panel 1. All mice were female and were ovariectomized. A) Spleen counts and weights were variable among treatment groups with no significant differences. B) Images are representative of the flow cytometry analysis strategy with gates set on FMO. C) Results are summarized in graphs showing a small but significant reduction in absolute numbers of F4/80 + spleen cells in ERα short +E2 vs. ERαKO null +E2 mice, as well as a significant reduction in absolute numbers of CD19 $^{+}$ spleen cells in ERαKO short +E2 vs. WT NZM. Although there were trends towards other differences, there were overall not significant differences among the 4 groups with regard to spleen T cells, NK-T cells or NK cells.

3.4. Spleen cell subsets in ERαKO (short) NZM vs ERαKO (null) NZM mice

Total number of spleen cells and spleen weight were variable within the cohorts with no significant differences observed between groups, although there was a trend towards reduced spleen counts in ERα short +E2 mice (Fig. 4A). Although some spleens were enlarged, there was no association between spleen weight and survival or renal disease. There was a trend towards smaller spleens in ERα short NZM mice regardless of E2 status.

Spleen cells were isolated and stained with either of two panels for flow cytometry analysis. Standard markers were used to look for changes in spleen composition of macrophages, B cells, T cells, NK-T cells, NK cells, and DC subsets, as outlined in methods. Gates were set on FMO (Fluorescence Minus One) and the gating strategy is outlined in Fig. 4B. There was a small, but statistically significant decrease, in mature macrophages, as indicated by F4/80 positivity, in spleens from ERα short +E2 mice compared with ERα null +E2 mice (Fig. 4C). Consistent with the kidney cellular results, there was also a trend towards reduced percent and absolute number of B cells in spleen of ERα short +E2 mice compared to the other groups, and this reduction was statistically significant compared with WT NZM (both percent and absolute number). There were no significant differences in either absolute numbers (Fig. 4C) or percent (data not shown) of T cells, NK-T cells, or NK cells among the different groups of mice.

Given the compelling evidence for estrogen and ERα impacts on dendritic cell (DC) development and function (reviewed in Ref. [23]), including our previous reports of ERα modulation of TLR-stimulated pathways in both conventional and plasmacytoid DCs in lupus prone mice [24–26], we hypothesized that DCs would be most differentially affected in ERα deficient mice. A second flow cytometry panel was utilized to look at antigen presenting cells with markers to assess conventional DCs in the spleen: MHCII, CD11c, CD11b, and CD8a. There was a trend towards reduced absolute number (but not percent) of MHCII+ cells in ERα short mice regardless of E2 repletion, but a significant reduction in absolute number of MHCII+ cells and MHCII +CD11c+ cells was observed between ERα short +E2 mice vs ERα null +E2 mice (Fig. 5B). As in non-autoimmune mice, ERα signaling was dispensible for cDC development (absolute numbers) [27], since all mice were either missing E2 (all OVXed) and/or a full length ERα. Interestingly, in the ERα short +E2 mice only, there was a significant

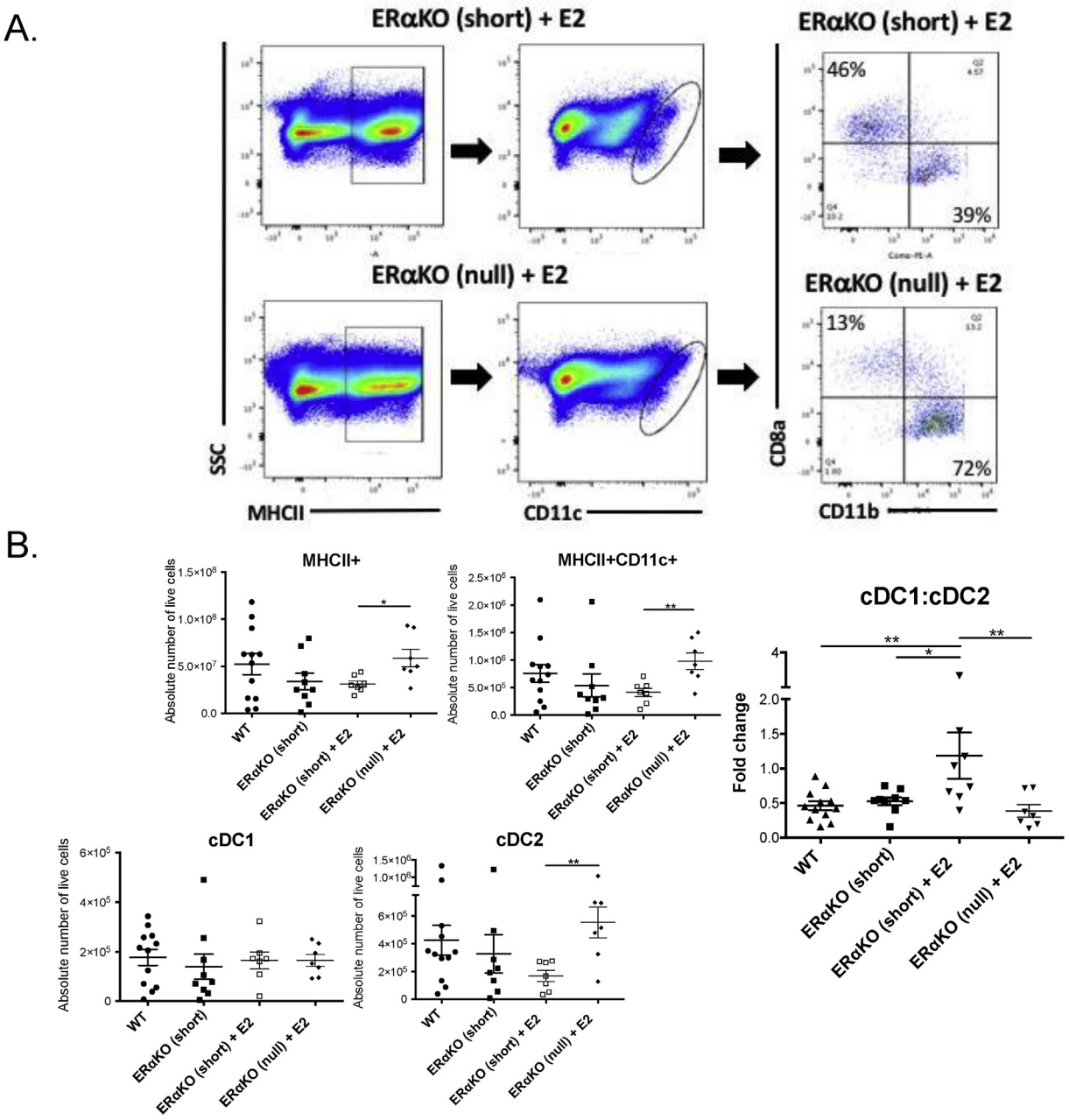

Fig. 5. Spleen cell flow cytometry analysis in ERαKO NZM2410 mice, Panel 2. A) Images are representative of the flow cytometry analysis of ERα short +E2 and ERα null +E2 mice demonstrating significant differences in DC number and subsets between the groups. B) Results are summarized in graphs below showing a significant reduction in the absolute number of MHCII+ cells in ERα short +E2, as well as in CD11c+ cells. CD11c+CD8a-CD11b+ (cDC2 cells) were significantly reduced in ERα short +E2 vs. ERα null +E2 mice. ERα short +E2 mice also had significantly higher ratio of CD8a+ to CD11b+ DCs (cDC1:cDC2 ratio) compared with all other groups.

decrease in cDC2 cells (MHCII+CD11c+CD8a-CD11b+), a DC subset that specializes in the presentation of antigen to $CD4^+$ T cells and favors polarization towards Th2 or Th17 responses [28,29]. There was no concomitant change in cDC1 cells (MHCII+CD11c+CD8a+CD11b-). An overall decrease in the number of mature, activated DCs in spleen of the ERα short +E2 group, also seen in the kidney, may partially explain the protected phenotype in this group. The short ERα (functionally an AF-1 mutant), in the setting of estrogen, impacts DC development more so than the presence or absence of full length ERα in this lupus model, in agreement with similar studies in non-autoimmune mice [27]. We additionally show for the first time that this short ERα plays a role in the development of specific DC subsets that likely impact lupus disease expression.

3.5. Flt3L-cultured BMDCs from ERαKO (short) NZM +E2 are profoundly impacted and differ in CD8a and CD11b positivity

Given the differences in cDC subsets observed in spleens of ERα short NZM mice, we looked at more proximal DC development by isolating bone marrow from femurs of NZM mice and culturing them *ex vivo* in Flt3L to enrich for DCs. In non-autoimmune mice, E2-ERα signaling inhibits the development of pDCs and cDCs in Flt3L-driven BM cultures [30]. In a separate study, the developmental effect on pDCs was dependent on ERα AF-1, while E2-ERα effects on cDCs were limited to functional endpoints (ex. activation markers, TLR responses), rather than reduction in absolute number [27].

In NZM lupus-prone mice, we found that BM yield (Supplemental

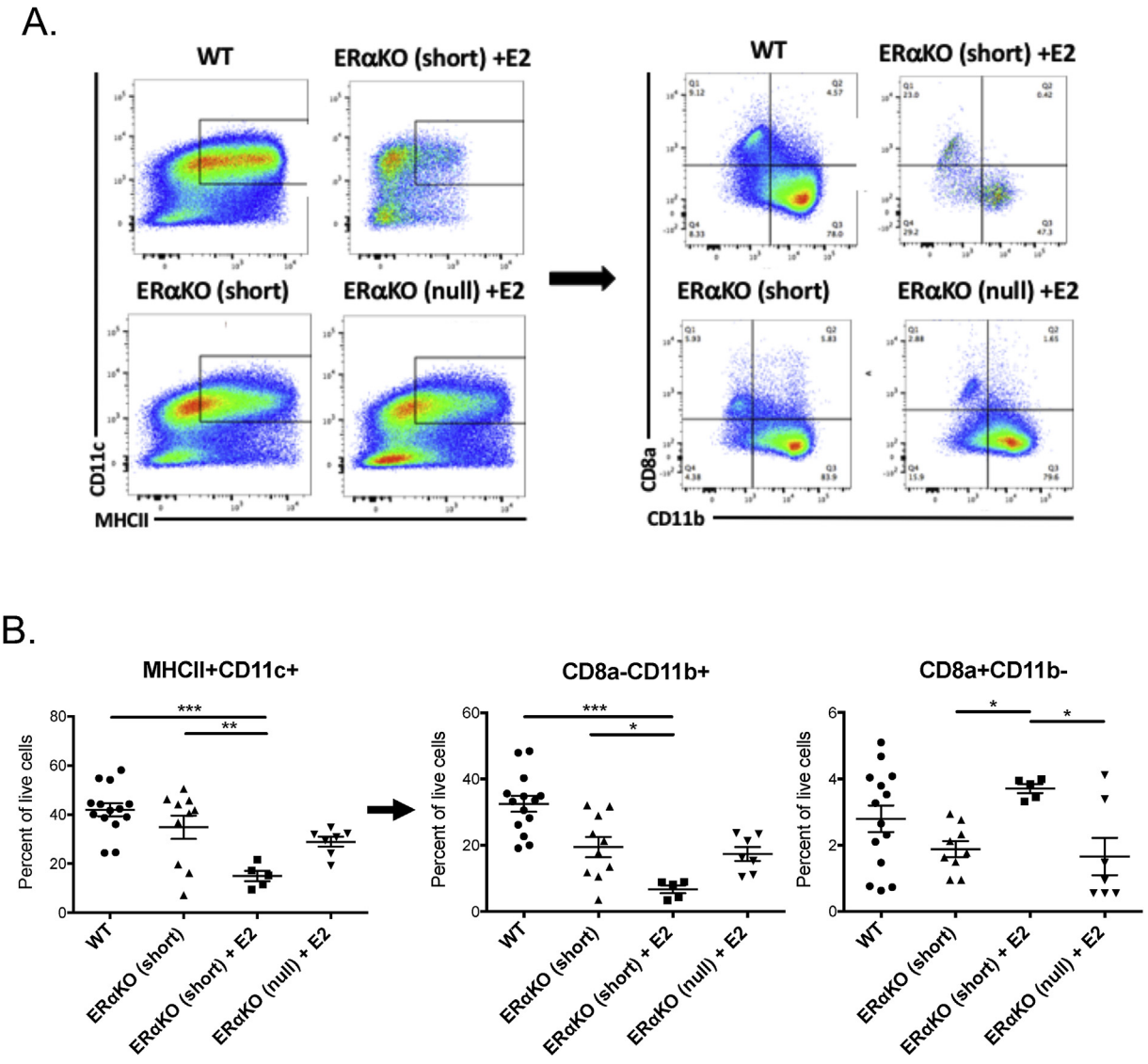

Fig. 6. Flt3L-cultured BM-DCs from ERα short NZM vs. WT NZM and ERα null NZM mice. BM from femurs of NZM mice was cultured *ex vivo* in Flt3L for 7d to enrich for DCs. MHCII + CD11c + cells were reduced in all strains without intact ERα, but ERα short NZM + E2 were the most profoundly impacted, both in bone marrow yield, as well as percent of DCs yielded. A) ERα short NZM + E2 mice had a significant reduction in number and percent of MHCII + CD11c + cells. B. Similar to spleens of these mice, cultured BM-DCs were also different in DC subset. After gating on MHCII + CD11c + live singlets, we observed a significant reduction in CD11b + DCs in ERα short NZM + E2 mice with a concomitant increase in CD8a + DCs.

Fig. 3) and percent of MHCII + CD11c + cells were both profoundly decreased in ERα short + E2 mice, with a significant difference in the percent of DCs yielded under these culture conditions (Fig. 6A and B). Similar to what we observed in spleens of the ERα short + E2 mice, Flt3L-cultured BMDCs also differed in DC subset numbers, with a significant reduction in CD11b + DCs (cDC2s) and a concomitant increase in CD11b-CD8a + cDC1s. Together these data suggest that both number and type of DC are impacted by E2 and ERα in lupus mice. Of note, all mice were OVXed, including WT, thus were missing either E2, or a full length ERα, or both. Consequently, these data suggest that in the presence of E2, the short ERα (AF-1 mutant) may actively suppress expansion of CD11b + (inflammatory) DCs, without similarly suppressing (or may even promote) CD8a + CD11b- DCs, since the effect of the liganded short ERα differs from both unliganded short ERα and E2-treated ERα null mice.

4. Discussion

To determine the impact of sex hormones and ERα deletion on murine lupus, we investigated the effect of ovariectomy (OVX) +/− estradiol (E2) repletion on lupus disease phenotype in NZM2410 mice. We compared ERα short (original Korach ERαKO) female mice, vs. ERα null (a total body ERα deletion mutant, also kind gift of Korach Lab). Unlike the NZM lupus prone mice with ERα null, which we recently described [12], ERα short mice were protected from lupus glomerulonephritis after OVX, if they were E2-repleted. ERα null mice were not protected regardless of estrogen repletion. Together with our previous work, these results support our working hypothesis that expression of the short form of ERα (that lacks AF-1) confers protection, and this effect requires E2. OVXed ERα short + E2 female mice lived significantly longer (all survived until the pre-determined sacrifice endpoint) vs. all other groups of female mice studied. This prolonged survival is similar to our previous report of the protective effect of ERα short in non-OVXed NZM2410 and MRL/lpr mice compared to wildtype [10].

Our current results rule out elevated testosterone as the protective effect in ERα short mice, since all animals were OVXed to control for hypergonadism. Without OVX, ERα deficiency leads to hypergonadism

with resultant aberrantly high serum testosterone and estrogen levels in both ERα short and ERα null females. These elevated levels of E2 and/or T2 were a potential confounder of previous experiments using ERαKO mice in lupus models. Supplemental Fig. 1 plots the elevated levels of E2 and T2 seen in un-manipulated female ERαKO mice and shows the normalized/reduced levels of T2 and E2 after OVX in mice from this study. As an additional control, the impact of E2 deficiency was assessed in WT and ERα short OVXed animals that were not E2-treated. In previous studies, elevated E2 levels seen in ERαKO animals may also be a confounder since high E2 levels could 1) act via an intact ERβ receptor to exert effects and/or 2) impact levels of other hormones such as prolactin which have immunomodulatory potential. We previously demonstrated that complete ERα deficiency is not directly protective in murine lupus [12]. OVXed vs. non-OVXed ERα null mice also have disparate disease states and immune phenotypes, likely due to aforementioned hypergonadism and elevated T2. Since all animals in this study were OVXed, our data demonstrates that neither E2 or T2 levels alone can explain the profound differences between ERα short +E2 mice and the other groups, particularly the ERα null +E2 mice, which have the same hormone profile, but were not protected. The protective effect is also not due to other intact nuclear hormone receptors (ERβ, AR, etc.), since these too are the same between ERα null +E2 and ERα short +E2 mice. Finally, and of equal import, the data demonstrate the requirement for E2 ligand in the protective effect, since both the truncated receptor *and* E2 are needed for the phenotype.

This study is the first report demonstrating a ligand-dependent protective role for a truncated ERα (AF-1 mutant) in murine lupus, after correcting for the confounder of aberrant hormone levels. Given that there exists an endogenously expressed short ERα variant in mice and humans, ERα46, that also lacks AF-1, these findings suggest that ERα46 may have an immune modulatory role, either directly or as a competitive inhibitor of full length ERα66. Thus, there is the possibility of targeting ERα by increasing ERα46 activity with a selective estrogen receptor modulator (SERM) for treating female-predominant autoimmune diseases. Pharmacological activation or inhibition of ERα already provides the foundation for therapeutic interventions in breast cancer and osteoporosis, for example. Since E2 was required for protection in this model, future work will focus on E2-induced expression levels of ERα variants in murine and human lupus, as well as possible effects of AF-2 mediated transcriptional activation in the setting of AF-1 absence. The appropriate use and efficacy of SERMs relies on our ability to understand the molecular actions of ERα in individual tissues and cell-types so we may uncouple beneficial effects from detrimental ones. Additional important studies include the factors that control expression of ERα46 versus ERα66 as perhaps increasing expression of ERα46 in immune cells may also be therapeutic.

Classic E2-dependent ERα activation involves both AF-1 and AF-2 interaction, but these activation domains can also independently activate transcription in a cell-type specific manner. For example, AF-1 is not required for E2-mediated vascular or bone protection, but is required for reproductive function and proliferation of uterine epithelial cells, as well as breast cancer cells [31–33]. It is additionally known that AF-1 and AF-2 transactivation depends on the differentiation stage of the cell. Specifically, while transcriptional activity of ERα relies on AF-1 in most estrogen target cell types, the less differentiated the cell, the less it mediates transcription through AF-1 [34]. AF-1 also appears to be a requirement for maturation of some immune cells. Seillet et al. demonstrated a differential requirement for ERα AF-1 in the development of different DC subsets *in vitro* when cultured in GM-CSF [27]. Specifically, AF-1 was required for functionally competent classic and plasmacytoid DCs. This was of great interest to us, since we previously showed a reduction in bone marrow-derived dendritic cell number in lupus-prone ERα short female mice, as well as a blunted response of these cells to TLR7/9 stimulation [24]. Thus, at some critical point in DC development (hypothesized to be at the macrophage-DC progenitor or common DC progenitor stage), absence of either E2 or an AF-1-intact ERα impedes development of a mature/activated (i.e. inflammatory) DC phenotype resulting in less TLR responsiveness. Conversely, in a lupus vulnerable genotype, the presence of E2 and an imbalance/shortage of protective ERα variants may promote maturation/activation of dysregulated DCs with heightened TLR responsiveness. Consistent with this, our protected lupus-prone ERα short +E2 mice, which express no full length ERα, have fewer mature inflammatory-type (MHCII+CD11c+CD11b+) DCs in the spleen and from Flt3L-cultured BM. This premise also makes sense within the framework of murine and human lupus, where DCs are known to be inappropriately over-activated and thus less capable of regulating autoreactive B cells [35].

While this study focused on controlling for aberrant sex hormone levels, as well as different ERα constructs, there were study design limitations to include 1) the length of time necessary to breed female ERα mutant mice for study participation in addition to the length of time to develop significant disease in this model (32 week endpoint). This resulted in inter-experimental time lapse between flow experiments (done variably on sacrifice days as animals came of age or became ill). To the extent possible, inter-experimental variables were minimized (antibody lots, consistent voltages on flow machine, etc). Where samples could be batch-processed (ex. anti-dsDNA ELISA, albumin ELISA, all histology), they were. An additional variable/limitation was the use of estradiol pellets for E2 replacement. Given the length of time to maintain E2-repletion (~6 months), daily E2 injections were not feasible due to time/labor constraints. We chose E2 pellets implanted subcutaneously instead of surgical implant of silastic tubing with E2 (both slow-release). Although both methods are validated for achieving stable plasma estradiol levels in murine studies, data support a reduced spike in serum E2 levels in the first week after pellet implant (vs. silastic tubing) [36]. The variability in serum estradiol levels seen in our E2-repleted mice is likely due to the estradiol assay itself. Although we used the estradiol ELISA method that is the gold standard at The Ligand Assay and Analysis Core of the Center for Research in Reproduction (CRR) at UVA, estradiol ELISAs, like IFNα ELISAs, have sensitivity and specificity issues [37]. We, and other investigators, have been working to optimize these assays over the years, however problems remain. Thus, we currently use serum estradiol levels *only* to demonstrate that we have corrected the supraphysiologic E2 levels that exist in female mice with hypergonadism (after OVX), as well as to confirm that mice with 17β-estradiol pellet-treatment have not been overdosed with supraphysiologic levels. For these purposes, the results of the serum E2 levels are still quite informative (similarly with the T2).

In summary, despite myriad studies of estrogen effects on different cell types in lupus, we still have limited understanding of the molecular mechanisms of action governing ERα and other nuclear hormone receptors in normal immunity and autoimmunity. Hormones clearly have variable effects on B, T, and dendritic cell functions [38]. Small clinical trials and case series have suggested benefits of androgens or known selective estrogen receptor modulators (SERMs) in lupus, but these are not well tolerated (ex. DHEA and fulvestrant, respectively) [39,40]. Other trials confirmed the safety of estrogen-containing oral contraceptives, although hormone-replacement therapy induced mild flares in post-menopausal lupus patients [41,42]. Overall, however, we have not progressed our understanding of sex effects on disease to the benefit of patients. The current study is the first to describe potential benefits of a short ERα (similar to endogenous ERα46 derived via an alternative transcription start site) in lupus. Presence alone of the short ERα, without ERα66, significantly impacts immune cells, particularly innate immune cells, in the NZM2410 lupus model, and profoundly reduces renal disease expression. Thus ERα46, and perhaps other endogenous ERα variants, represent a potential therapeutic target based on its apparent potential anti-inflammatory effects. Future studies are needed to determine whether imbalance of ERα variants plays a role in disease, and to identify the central regulatory functions of ERα46 (vs. the classic full-length ERα66) on gene targets, as well as AF-1- vs. AF-2-mediated

transcription. In addition, given that most autoimmune diseases are more prevalent in women, these studies may help identify the mechanisms of estrogen and ERα action that lead to increased female risk in autoimmunity.

Author contributions

MC and GG directed the work, contributed to designing the study, and reviewed/interpreted the data. JS helped with data analysis. MC and JW conducted all experiments with help from MLR (related *in vitro* studies), JE (breeding, genotyping, bleeding and urine collection), and PR (pathology). MC and JW prepared the manuscript and figures. GG contributed to manuscript revisions. All authors read and approved the final manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.10.011.

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