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Complete knockout of estrogen receptor alpha is not directly protective in murine lupus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic and potentially severe autoimmune disease that disproportionately affects women. Despite a known role for hormonal factors impacting autoimmunity and disease pathogenesis, the specific mechanisms of action remain poorly understood. Our laboratory previously backcrossed "estrogen receptor alpha knockout (ERαKO)" mice onto the NZM2410 lupus prone background to generate NZM/ERαKO mice. This original ERαKO mouse, developed in the mid-1990s and utilized in hundreds of published studies, is not in fact ERα null. They express an N-terminally truncated ERα, and are considered a functional KO. They have physiologic deficiencies including infertility due to disruption of a critical activation domain (AF-1) at the N terminus of ERα, required for most classic estrogen (E2) actions. We demonstrated that female NZM/ERαKO mice had significantly less renal disease and significantly prolonged survival compared to WT littermates despite similar serum levels of autoantibodies and glomerular immune complex deposition. Herein, we present results of experiments using a lupus prone true ERα −/− mice (deletional KO mice on the NZM2410 background), surprisingly finding that these animals were not protected if they were ovariectomized, even if E2-repleted, suggesting that another hormonal component confers protection, possibly testosterone, rather than the absence of the full-length ERα.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by production of autoantibodies and immune complex-mediated end-organ damage. Nine out of ten patients diagnosed with lupus 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 strong epidemiologic data to support a role for estrogen impact on disease since the incidence of disease is highest during reproductive years when women are most hormonally active. This is in contrast to pre-menarche and post-menopause, when lupus incidence is lower and the female to male ratio is much less profound. Estrogens, mainly via ERα, may promote lupus by facilitating loss of immunologic tolerance and enhancing production of autoantibodies [1,2]. In recent years, other immune effects of estrogen were identified, including modulation of Toll-like receptor (TLR) pathways and dendritic cell development that both play a significant role in lupus pathogenesis [3,4]. The molecular pathways through which estrogens exert these effects are not fully defined.

In murine models of lupus, manipulation of sex hormones has significant impact on disease expression. NZB/NZW mice and some of the derived NZM strains (i.e. NZM2328) have significant female predominance of disease [5]. Both genders of MRL/lpr and NZM2410 mice develop severe lupus, however, there is a trend towards earlier and more severe disease in females [6]. In classic experiments by Roubinian et al., and later by Tarkowski et al., manipulation of sex hormones and castration led to significant effects on disease expression [7–12]. Specifically, castration of male NZB/NZW or MRL/lpr mice led to female-like disease, and ovariectomy with androgen replacement in female mice led to disease protection. Administration of pharmacologic doses of estrogen led to significant enhancement of disease in ovariectomized (OVX) females, castrated males, and un-manipulated females [12].

Estrogen has pleiotropic effects on many different cell types, including immune cells. Thus, estrogens do not act in the same manner in all inflammatory diseases. Even within a disease, estrogen may play both anti-inflammatory and pro-inflammatory roles depending on the disease state, the target organ, the type of immune stimulus, the length of exposure and the presence of other hormones and their receptors. This is consistent with the immense variability in immune responses necessary throughout a female's reproductive life. Females are known to respond more vigorously to infection and vaccination, but are unfortunately more susceptible to autoimmunity. Testosterone (T2) also plays a role in sex-based disparity in immune responses. Multiple

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lines of study have demonstrated immune-suppressive effects of androgens on both adaptive and innate immune responses (Reviewed in [13]).

This study was undertaken to clarify unanswered questions from previous studies done using ERαKO animals. The original ERαKO mice express an N-terminally truncated ERα that includes a disruption of a critical activation domain (AF-1) resulting in, strictly speaking, a functional knockout of ERα. They are infertile and have physiologic deficiencies wherever classic estrogen action via AF-1 is required (ex. reproductive tissues) [14]. However, these animals still express an ERα protein with an intact DNA-binding domain (DBD), ligand binding domain (LBD) and AF-2, and may retain some non-classical functions [15]. Thus, they have the potential for impacting the murine lupus phenotype differently than a complete ERα knockout. Additionally, both the functional knockout (ERαKO) and complete knockout (ERα −/−) mice have hypergonadism (supra-physiologic serum levels of estrogen and testosterone, as a result of ERα dysfunction/deletion) which can be immunomodulatory. In this study, we compared intact/unmanipulated NZM ERα −/− mice to ovariectomized NZM ERα −/− mice (E2 and T2 depleted). We also included a group of ovariectomized NZM ERα −/− mice with repletion of E2 via pellet, to determine estrogen impact on the lupus phenotype in the setting of ERα −/− (since E2 may also have immune effects via ERβ, ERR or non-ligand bound functions).

The goal of this study was to assess whether complete deficiency of ERα is similarly protective in lupus disease expression as the functional knockout. We additionally determined the immuno-phenotype of these animals. Our results reveal that the complete ERα knockout is not directly protective since ovariectomized (OVX'd) ERα −/− mice are not protected. Like the NZM ERαKO mice, unmanipulated NZM ERα −/− mice are protected. With removal of sex hormones via OVX, there is no effect of ERα −/−, thus, this study suggests that elevated testosterone (in this case, secondary to hypergonadism) confers protection in the setting of ERα −/−, rather than deficiency of the full length receptor itself. Further study with T2 treatment of the OVX'd NZM ERα −/− group would be required to confirm that androgen treatment, as in historical studies, confers a protective phenotype. This report is of clinical significance since it corrects a previously held belief that ERα and E2 are generally pro-inflammatory in females and lupus murine models, when in fact there is evidence that E2 can have anti-inflammatory effects in specific settings. Regardless, improved understanding of sex hormones and nuclear hormone receptor action (ER and AR) may enable therapeutic targeting of specific functions, as opposed to general manipulation of hormones, to allow for separation of hormone effects on different tissues (immune cells versus reproductive tissue, for example).

2. Materials and methods

2.1. Mice

Mice were maintained at the Ralph H. Johnson VAMC Animal Facility (Charleston, SC). Animal protocols followed the principles outlined in the Guide for the Care and Use of Laboratory Animals, and were approved by MUSC's and the VA's IACUC. The NZM2410 mice were acquired from Jackson Laboratory (Bar Harbor, ME, USA), the ERα −/− C57BL/6 mouse strain was a kind gift of Dr. Ken Korach. The two strains were backcrossed for >10 generations to create the ERα −/− NZM2410 mouse (NZM ERα −/−). They were maintained on a 12 h light/dark cycle with access to food and water ad libitum. All experimental mice (n = 51) were female and were littermates when possible. Two cohorts (NZM WT and NZM ERα −/−) were unmanipulated. All other mice were ovariectomized (OVX) at 4 weeks of age, before puberty, and 2 groups subsequently received 0.1 mg, 90-day sustained release 17β-estradiol pellet, implanted sub-dermally (Innovative Research of America, Sarasota, FL, USA). Mice were sacrificed by cervical dislocation following induction of anesthesia by isoflurane at 32 weeks of age or when they reached pre-determined sacrifice requirements (>10% loss of weight, >500 mg urine protein as assessed by dipstick, or upon recommendation by the animal facility veterinarian).

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

Serum was collected throughout the experiment at 2–4 week intervals and at time of sacrifice via submandibular bleed. Serum anti-dsDNA was measured by ELISA assay as previously described [6]. 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. Kidney processing and renal pathology

One kidney was divided evenly for renal pathology and immunofluorescent analysis (IF). One half was snap frozen in liquid nitrogen and stored at −80 °C for IF analysis, the other half was fixed with buffered formalin, embedded in paraffin, and then sectioned and stained with hematoxylin and eosin. 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 glomerular pathology score as previously described [17]. Deposition of IgG and complement component C3 was assessed by immunofluorescence after incubating slides with rabbit anti-mouse IgG FITC (MP Biomedical) and sheep anti-mouse C3 FITC (MP Biomedical). IgG and C3 were graded 0–3 for intensity of staining as previously described [17]. [A second kidney was processed for flow cytometry staining. The outer membrane of the kidney was removed before being sliced into pieces and digested with DNase I (Roche Life Sciences, Indianapolis, Indiana) and collagenase IV (Sigma Aldrich, St. Louis, MO) for 30 min at 37C on a shaker. The kidney was then put through a 70 μm strainer and PBMCs were isolated using a Percoll gradient (Sigma Aldrich, St. Louis, MO). PBMCs were washed 2× with PBS before staining for flow cytometry.]

2.5. Spleen flow cytometry

Spleens were harvested and kept in complete RPMI media (10% fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin) on ice during processing. The spleens were processed through 40um strainers and depleted of red blood cells with red blood cell lysis buffer (144 mM NH_4Cl and 17 mM Tris, pH 7.6). Cells were washed twice with cold complete RPMI before being stained for flow cytometry analysis. Spleen cells (4×10^6 per sample) were resuspended in staining buffer (0.5% BSA and 0.02% sodium azide in 1 × PBS). 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 stained with Panel I: F4/80-Brillaint violet 421 (1:100), CD19-PerCP/Cy5.5 (1:100), CD3-Brilliant violet 605 (1:100), 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