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Roles of activating functions 1 and 2 of estrogen receptor α in lymphopoiesis

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Abstract

Apart from the role of sex steroids in reproduction, sex steroids are also important regulators of the immune system. 17 β -estradiol (E2) represses T and B cell development, but augments B cell function, possibly explaining the different nature of immune responses in men and women. Both E2 and selective estrogen receptors modulators (SERM) act via estrogen receptors (ER). Activating functions (AF)-1 and 2 of the ER bind to coregulators and thus influence target gene transcription and subsequent cellular response to ER activation. The importance of ER α AF-1 and AF-2 in the immunomodulatory effects of E2/SERM has previously not been reported. Thus, detailed studies of T and B lymphopoiesis were performed in ovariectomized E2-, lasofoxifene- or raloxifene-treated mice lacking either AF-1 or AF-2 domains of ER α , and their wild-type littermate controls. Immune cell phenotypes were analyzed with flow cytometry. All E2 and SERM-mediated inhibitory effects on thymus cellularity and thymic T cell development were clearly dependent on both ER α AFs. Interestingly, divergent roles of ER α AF-1 and ER α AF-2 in E2 and SERM-mediated modulation of bone marrow B lymphopoiesis were found. In contrast to E2, effects of lasofoxifene on early B cells did not require functional ER α AF-2, while ER α AF-1 was indispensable. Raloxifene reduced early B cells partly independent of both ER α AF-1 and ER α AF-2. Results from this study increase the understanding of the impact of ER modulation on the immune system, which can be useful in the clarification of the molecular actions of SERMs and in the development of new SERM.

Key Words

- ▶ lymphopoiesis
- ▶ estrogen receptor alpha
- ▶ estradiol
- ▶ selective estrogen receptor modulators

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Introduction

Besides the major roles of estrogens in reproduction and bone health, estrogens have strong impact on the immune system (Straub 2007). In general, estrogens inhibit thymus size and hamper T and B lymphopoiesis, in

thymus and bone marrow respectively, while enhancing peripheral B cell effector function in terms of antibody production (Medina & Kincade 1994, Rijhsinghani *et al.* 1996, Erlandsson *et al.* 2002). In addition, estrogens also

influence autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (Yung 1999, D'Elia et al. 2003).

Two receptors are mainly responsible for the actions of estrogens; estrogen receptor (ER) α and β , both expressed by cells of the immune system (Straub 2007). The ERs are ligand-activated nuclear transcription factors, which bind to certain DNA sequences called estrogen response elements (ERE), resulting in induction or repression of transcription of target genes. This mode of action is further complicated by, and dependent on, the binding of coregulators (coactivators or corepressors) to the receptor–ligand complex. Coregulators are recruited to specific domains of the ER protein named activating functions (AF)-1 and 2, subsequently enabling or disabling target gene transcription, decided by the properties of the recruited coregulators (Tora et al. 1989). The AF-1 and AF-2 domains can act either independently, or in synergy, to achieve complete ligand-dependent transcriptional activity of ERs (Tzukerman et al. 1994). As nuclear receptors, ERs contain a ligand-binding domain (LBD) and a DNA-binding domain (DBD). The LBD is located in the C-terminal part of the ER protein, where also AF-2 is found, thus being ligand dependent (Tora et al. 1989). The ligand-independent AF-1 domain is situated within the N-terminal part of the ER protein (Tora et al. 1989).

Selective estrogen receptor modulators (SERMs) are a group of pharmacological compounds designed as tailor-made mixed ER agonists and/or antagonists. The SERMs raloxifene and lasofoxifene were developed as treatments for postmenopausal bone loss and in addition, raloxifene is used in breast cancer prevention due to its ER antagonistic actions in breast tissue (Delmas et al. 1997, Cummings et al. 1999, 2010). SERMs alter the conformation of the ER, thus modifying the ligand-binding activity. However, the tissue-specific ER actions of SERMs are determined by levels, localization and activity of coactivators and corepressors and relative expression of ER α / β in target cells (Shang and Brown 2002, Nelson et al. 2013). The ER antagonistic action of raloxifene is due to an altered conformation of the LBD where AF-2 is prevented from binding coactivators (Brzozowski et al. 1997). Also, the finding that mutations in AF-2 convert a pure ER antagonistic SERM into an ER agonist supports the critical role for AFs in SERM activity (Arao et al. 2011, Moverare-Skrtic et al. 2014). Since coregulators, with the potential to bind to AF-1 and AF-2, are crucial in the cell-specific effects of SERMs, studies of these activating functions of the ER can provide us with new insights into SERM mechanisms of action.

We have previously elucidated the role of ER α AF-1 and ER α AF-2 in E2- and SERM-mediated skeletal maintenance after ovariectomy, by studying mice lacking either AF-1 (ER α AF-1⁰) or AF-2 (ER α AF-2⁰) (Borjesson et al. 2011, 2016, Moverare-Skrtic et al. 2014). These experiments demonstrated that ER α AF-2 is essential for the majority of the classical E2-mediated effects including preservation of cortical and trabecular bone microarchitecture, reduction in thymus size and increase in uterus size (Borjesson et al. 2011). In contrast, the necessity for ER α AF-1 is tissue specific, and this domain is crucial for E2-mediated effects in trabecular bone and in uterus, but dispensable for effects in cortical bone (Borjesson et al. 2011). However, anti-osteoporotic effects of SERMs in ovariectomized mice are dependent on the presence of both ER α AF-1 as well as ER α AF-2 (Moverare-Skrtic et al. 2014, Borjesson et al. 2016). An overview of the phenotypes of the ER α AF-1⁰ and ER α AF-2⁰ mice is found in a recent review (Arnal et al. 2017).

Thorough studies have already clarified specific effects of E2, raloxifene and lasofoxifene on adaptive immune development (Bernardi et al. 2014, 2015). However, the roles of ER α AF-1 and ER α AF-2 in the immunomodulating effects of SERMs or E2 have not been studied on a cellular level. Thus, this study is the first of its kind, revealing in detail whether ER α AF-1 and ER α AF-2 are necessary for the E2- and SERM-mediated effects on development of B and T cells. Results from this study increase the understanding of the impact of ER modulation on the immune system and can be useful in the development of new SERMs with reduced side effects.

Materials and methods

Animals and treatments

The regional Ethical Review Board in Gothenburg approved all experimental procedures in the study. Mice were kept under standard environmental conditions and fed with laboratory chow and tap water *ad libitum*. ER α AF-1⁰ and ER α AF-2⁰ mice and their littermate WT controls, which are inbred on a C57BL/6 background, were generated by breeding heterozygous females and males. The generation of ER α AF-1⁰ and ER α AF-2⁰ mice has been described in detail elsewhere (Billon-Gales et al. 2009, Borjesson et al. 2011). Briefly, the ER α AF-1⁰ mice have a specific deletion of AF-1 (amino acids 2–148) and therefore express the truncated 49-kDa ER α protein and also express the physiologically occurring but less abundantly expressed

Figure 1

Illustration of the ER α protein. The ER α structure is divided into six functional domains, A-F (Green et al. 1986). The activation function-1 (AF-1) is located within the A/B domains in the N-terminus, and the ER α AF-1⁰ mice have deletions in this domain, lacking amino acids 2–148. The C domain contains the DNA-binding domain (DBD). The D domain contains a hinge region and the nuclear localization signal. AF-2 is located in the ligand-binding domain (LBD), in the E/F domains of the C-terminus. ER α AF-2⁰ mice lack amino acids 543–549 of ER α .

46-kDa ER α isoform, instead of the full-length 66-kDa ER α protein. ER α AF-2⁰ mice have a deletion in the AF-2 core, which corresponds to amino acids 543–549. A schematic illustration over the ER α protein with the specific deletions in the ER α AF-1⁰ and ER α AF-2⁰ mice is shown in Fig. 1.

Ovariectomy (OVX) was performed in 8-week-old mice, as described previously (Andersson et al. 2015). After 1 week of recovery, treatment was started with subcutaneous injections 5 days/week with vehicle (veh; Miglyol 812; OmyaPeralta, Hamburg, Germany), 17 β -estradiol-3-benzoate (E2; 1 μ g/day; Sigma-Aldrich), raloxifene (RAL; 60 or 120 μ g/day; Sigma-Aldrich) or lasofoxifene (LAS; 4 or 8 μ g/day; a kind gift from Pfizer). The higher doses of RAL and LAS were used in the ER α AF-2⁰ mice and their littermate WT controls, while the lower doses were used in ER α AF-1⁰ mice and their littermate WT controls. After 3 weeks of treatment, mice were anesthetized with ketamine (Pfizer) and dexdomitor (Orion, Espoo, Finland), and killed by cervical dislocation. Doses of E2 and SERMs used in this study have previously been used in osteoporosis studies in OVX mice (Moverare-Skrtic et al. 2014, Borjesson et al. 2016). Body surface area calculations ensured that SERM doses used in mice were corresponding to human doses (Cummings et al. 1999, 2010, Reagan-Shaw et al. 2008).

Flow cytometry

Bone marrow (BM) cells were obtained by flushing femurs and/or humerus with PBS, and erythrocytes were lysed. Thymi were pressed through 70 μ m cell strainers to obtain single-cell suspensions. Cellularity was determined using an automated cell counter (Sysmex, Norderstedt, Germany). Purified rat anti-mouse CD16/32 (BD Biosciences, Franklin Lakes, NJ, USA) was added

to block antibodies from binding to Fc receptors, prior staining of surface markers. Cells were stained with the following fluorochrome-conjugated antibodies; anti-mouse CD8a PE (clone 53–6.7, Biolegend, San Diego, CA, USA), anti-mouse CD4 BD Horizon V450 (clone RM4–5, BD), anti-mouse CD117/ckit APC (clone 2B8, Biolegend), anti-mouse CD45R/B220 PerCP (clone RA3-6B2, Biolegend), anti-mouse CD19 BD Horizon V450 (clone 1D3, BD), anti-mouse IgM PE (clone 1B4B1, Southern Biotechnology, Birmingham, AL, USA) and anti-mouse CD93 PE-Cy7 (clone AA4.1, Biolegend). Fluorescence-minus-one (FMO)-stained samples were used as controls. Data were acquired on a BD FACS Canto II and analyzed using Flow Jo 8.8.6 (Three Star, Ashland, USA).

Statistical analysis

Statistical analyses were conducted in SPSS software, version 21 (IBM). Normal distribution of data was assessed and when needed, data were logarithmically transformed to increase normality. ANOVA was used unless day-to-day variation was found, and then ANCOVA was used instead with termination day designated as cofactor. Within each genotype, treatment groups were compared with vehicle group by using Dunnet's *t post hoc* test or Dunnet's T3 *post hoc* test if Levene's test revealed unequal variances between groups. Results are presented as arithmetic mean $\pm$ S.E.M. and *P* values <0.05 were considered statistically significant.

Results

Estrogenic effects on thymic T cell development require functional AF-1 and AF-2 domains of ER α

Ovariectomized (OVX) WT or littermate OVX ER α AF-1⁰ and ER α AF-2⁰ mice were treated daily with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle (veh), (the higher doses of LAS and RAL used in the ER α AF-2⁰ experiment). Thymi were excised after three weeks of treatment. In accordance with previous studies, we found that E2 dramatically decreased thymus cellularity in WT mice (Fig. 2A and B) (Rijhsinghani et al. 1996). SERMs also reduced thymus cellularity, although much weaker than E2 (Fig. 2A and B) (Bernardi et al. 2015). Reductions in thymus cellularity by both E2 and SERMs were dependent on the presence of both AF-1 and AF-2 domains of the ER α protein since thymus cellularity in ER α AF-1⁰ and ER α AF-2⁰ mice were completely unaffected by all treatments (Fig. 2A and B).

Figure 2

E2 does not influence thymus cellularity in ER α AF-1- and ER α AF-2-deficient mice. OVX ER α AF-1⁰, ER α AF-2⁰ and corresponding littermate wild-type (WT) control mice were treated with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 μ g or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle for 5 days/week during 3 weeks, with the higher doses of LAS and RAL used in the ER α AF-2⁰ mice and their corresponding WT controls. Thymi were dissected at termination and total thymus cellularity was assessed by an automated cell counter, in (A) ER α AF-1⁰ and WT controls and (B) ER α AF-2⁰ and WT controls. Statistical differences between vehicle and treatment groups were analyzed within each genotype, with ANCOVA (termination day as covariate) except for A (KO) where ANOVA was used, followed by Dunnett's *t* post hoc test. Mean $\pm$ S.E.M. is shown, $n=8-11$ mice/group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs vehicle.

Effects on thymus weights after treatment with RAL, LAS and E2 in ER α AF-1⁰ and ER α AF-2⁰ mice have been reported elsewhere; both E2 and SERM decreased thymus weight in WT but not in ER α AF-1⁰ mice or ER α AF-2⁰ mice (Moverare-Skrtic et al. 2014, Borjesson et al. 2016).

Lymphoid progenitor cells migrate from bone marrow to thymus where T cell maturation occurs; from T cell receptor-expressing double-positive (DP) immature CD4⁺CD8⁺ T cells into mature naïve CD4⁺ or CD8⁺ single-positive (SP) T cells, which exit the thymus. These stages of developing T cells were characterized by flow cytometry as depicted in Fig. 3A. Numbers of thymic DP cells were decreased by E2 and SERM in WT mice (Fig. 3B and C); however, this reduction was not significant after RAL treatment in WT littermates to ER α AF-1⁰ mice (Fig. 3B). No effects of the treatments were found on the number of DP cells in ER α AF-1⁰ and ER α AF-2⁰ mice. In the process of positive and negative selection, which is dependent on recognition of MHC I or MHC II, DP cells downregulate either CD4 or CD8 and become SP cells. Both CD4 and CD8 SP T cells were decreased by E2 treatment in WT mice in these experiments; effects that required the presence of both AF-1 and AF-2 (Fig. 3D, E, F and G). LAS also reduced levels of SP CD4⁺ and SP CD8⁺ cells in the WT controls but not in ER α AF-1⁰ mice, otherwise SERMs in general lacked effects on SP cells. In summary, the AF-1 and AF-2 domains of ER α are necessary for classical estrogenic effects on T cell development in thymus.

Effects of E2 on bone marrow CD19⁺ B cells are dependent on ER α AF-2 but not ER α AF-1

The bone marrow cellular compartment diminishes after E2 treatment, possibly due to both enhanced endosteal bone formation as well as suppression of B lymphopoiesis. Thus, total bone marrow cellularity in WT mice were inhibited by E2 as well as by lasofoxifene, but to a lower extent by raloxifene. These inhibitory effects were completely absent in mice lacking AF-1 or AF-2 (Fig. 4A and B; data from vehicle and E2 groups in Fig. 4B have been published before (Moverare-Skrtic et al. 2014)). Furthermore, the total proportion of CD19⁺ B cells in bone marrow was decreased by E2, which was dependent on AF-2 but not on AF-1 (Fig. 4C and D). The SERMs lacked effects on the CD19⁺ B cell population.

Divergent roles of ER α AF-1 and ER α AF-2 in E2- and SERM-mediated modulation of B lymphopoiesis

B cell development in bone marrow results in cells expressing the B cell antigen receptor (BCR). Heavy- and light-chain Ig genes are rearranged at the progenitor B (pro-B) and precursor B (pre-B) cell stages, and IgM is expressed at the cell surface in the immature stage. Immature B cells leave the bone marrow for further activation and differentiation

Figure 3

Functional ER α AF-1 and ER α AF-2 are required for estrogenic effects on thymic T cell development. OVX ER α AF-1⁰, ER α AF-2⁰ and corresponding littermate wild-type (WT) control mice were treated with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 μ g or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle for 5 days/week during 3 weeks, with the higher doses of LAS and RAL used in the ER α AF-2⁰ mice and their corresponding WT controls. Cells from thymus were analyzed by flow cytometry and absolute numbers of cells were calculated by multiplying the frequencies obtained by flow cytometry with total thymus cellularity obtained by an automated cell counter. Representative graph showing gating strategies for assessing developmental stages of T lymphopoiesis (A). Number of double-positive (DP) CD4⁺CD8⁺ cells in ER α AF-1⁰ mice (B), ER α AF-2⁰ mice (C) and corresponding WT controls. Number of single-positive (SP) CD4⁺ cells in ER α AF-1⁰ mice (D), ER α AF-2⁰ mice (E) and corresponding WT controls. Number of single-positive CD8⁺ cells in ER α AF-1⁰ mice (F), ER α AF-2⁰ mice (G) and corresponding WT controls. Statistical differences between vehicle and treatment groups were analyzed within each genotype, with ANOVA, except from data in (D: WT) which were analyzed with ANCOVA (termination day as covariate). Dunnett's *t* post hoc test was used except when unequal variances were found as in (E: both; F: both; G: KO), then Dunnett's T3 post hoc test was used. Mean $\pm$ s.e.m. is shown, $n=7-10$ mice/group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs vehicle.

in secondary lymphoid organs. We have previously characterized the suppressive actions of E2 and SERM on B lymphopoiesis in bone marrow (Bernardi et al. 2014). Pro-B cells (B220^{low}ckit⁺CD19⁻) and pre-BI (B220^{low}ckit⁺CD19⁺) populations (Osmond et al. 1998) were analyzed as shown in Fig. 5A. E2 did not significantly influence the proportions of pro-B cells; however, both SERMs decreased pro-B cells in the WT mice in the ER α AF-2⁰ experiment, but not in the ER α AF-1⁰ experiment (possibly due to the lower SERM doses used in the ER α AF-1⁰ experiment) (Fig. 5B and C).

Nevertheless, the SERM-induced decreases in pro-B cells were dependent on functional AF-2 (Fig. 5B and C). Proportions of pre-BI cells were dramatically suppressed by E2 and SERMs (Fig. 5D and E). E2-induced suppression of the pre-BI population required functional AF-2 but not AF-1 (Fig. 5D and E). As opposed to E2, effects of LAS on pre-BI cells did not require functional AF-2, instead AF-1 was indispensable for this effect (Fig. 5D and E). Effects of RAL on pre-BI cells were weakened, but not abolished, in mice lacking AF-1 and AF-2 (Fig. 5D and E).

Figure 4

Roles of functional ER α AF-1 and ER α AF-2 in estrogenic effects on bone marrow cellularity and total B cell population. OVX ER α AF-1⁰, ER α AF-2⁰ and corresponding littermate wild-type (WT) control mice were treated with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 μ g or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle for 5 days/week during 3 weeks, with the higher doses of LAS and RAL used in the ER α AF-2⁰ mice and their corresponding WT controls. Total bone marrow cellularity (obtained from humerus and femur) was assessed by an automated cell counter, in (A) ER α AF-1⁰ and WT controls and (B) ER α AF-2⁰ and WT controls. Results from vehicle and E2-treated groups in (B) have previously been published elsewhere (Moverare-Skrtec et al. 2014). Cells from bone marrow were further analyzed by flow cytometry, and frequency of total CD19⁺ B cell population (of lymphocyte gate) was assessed in ER α AF-1⁰ mice (C), ER α AF-2⁰ mice (D) and corresponding WT controls. Statistical differences between vehicle and treatment groups were analyzed within each genotype, with ANOVA, except from data in (A: WT; B: WT; C: WT; D: KO) which were analyzed with ANCOVA (termination day as covariate). Dunnett's *t post hoc* test was used except when unequal variances were found as in (C: WT), then Dunnett's T3 *post hoc* test was used. Mean $\pm$ S.E.M. is shown, $n=8-11$ mice/group. ** $P<0.01$, *** $P<0.001$ vs vehicle.

The last developmental stage in B lymphopoiesis before cells leave bone marrow – B220^{low}IgM⁺ immature B cells – was analyzed as shown in representative flow cytometry plots in Fig. 6A. The immature B cell population is also described to express CD93 (Rolink et al. 1998), and the majority of cells within the B220^{low}IgM⁺ population also did so (data not shown). E2 reduced the proportion of immature B cells in WT mice (Fig. 6B), although the effect did not reach statistical significance in WT littermate controls to ER α AF-2⁰ mice (Fig. 6C). The E2 effects were dependent on functional AF-2 (Fig. 6C), but not AF-1 (Fig. 6B). The SERMs did not influence the immature B cell population (Fig. 6B and C). B220^{high}CD93⁺ B cells (Fig. 6D) represent a population of mature B cells in bone marrow, including for example, recirculating long-lived follicular B cells (Rolink et al. 1998). B220^{high}CD93⁺ B cells were slightly increased in proportion after E2 treatment, an effect that was independent of functional ER α AF-2 (Fig. 6F). LAS did not influence the mature B cell population; however, RAL increased this population, and AF-2, but not AF-1, was required for the effect of RAL (Fig. 6E and F). To conclude, both E2 and SERMs regulate B lymphopoiesis and do to some extent require divergent ER domains in terms of necessity for AF-1 or AF-2 for these actions.

Discussion

Estrogens regulate immune responses, providing one explanatory factor for the difference between men and women regarding susceptibility to autoimmune diseases and infections (Klein & Flanagan 2016). This study presents several novel insights into estrogen receptor-mediated regulation of the adaptive immune system, clearly revealing the role of functional AF-1 or AF-2 of ER α in the effects of E2 and SERMs, on the development of T and B lymphocytes in mice.

Thymus involutes after estradiol treatment, or during pregnancy, in both mice and humans (Marotti et al. 1984, Clarke & Kendall 1994, Rijhsinghani et al. 1996). E2-induced reduction in thymus cellularity is primarily due to a dramatic decrease of immature T cells and due to this inhibition, the frequencies of SP T cells are relatively increased after E2 administration (Zoller & Kersh 2006). In addition, E2 reduces the number of cortical thymic epithelial cells, and the cortex area of thymus is diminished (Erlandsson et al. 2000). Effects of E2 on thymus weight are absent in total ER α ^{-/-} mice, as well as in the ER α AF-1⁰ and ER α AF-2⁰ mice (Borjesson et al. 2011). Our study reveals that ER α AF-1 and ER α AF-2 are

Figure 5

Roles of functional ER α AF-1 and ER α AF-2 in ER-mediated alterations in early B lymphopoiesis. OVX ER α AF-1⁰, ER α AF-2⁰ and corresponding littermate wild-type (WT) control mice were treated with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 μ g or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle for 5 days/week during 3 weeks, with the higher doses of LAS and RAL used in the ER α AF-2⁰ mice and their corresponding WT controls. (A) Representative graph showing gating strategies for assessing developmental stages of early B lymphopoiesis in bone marrow. Frequency of pro-B cells (B220^{low}ckit⁺CD19⁻) cells in (B) ER α AF-1⁰ mice and (C) ER α AF-2⁰ mice and corresponding WT controls. Frequency of pre-BI (B220^{low}ckit⁺CD19⁺) in (D) ER α AF-1⁰ mice, (E) ER α AF-2⁰ mice and corresponding WT controls. All populations are expressed as frequency of parent lymphocyte gate. Statistical differences between vehicle and treatment groups were analyzed within each genotype, with ANCOVA (termination day as covariate), except from data in (C: both groups; D: WT) which were analyzed with ANOVA. Dunnett's *t* post hoc test was used except when unequal variances were found as in (C: WT), then Dunnett's T3 post hoc test was used. Mean $\pm$ S.E.M. is shown, $n=7-10$ mice/group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs vehicle.

also necessary for the E2-induced effects on the distinct developmental stages of T cells in thymus.

In comparison to E2, SERMs exerted moderate effects on developing T cells in WT mice, although in this study, the effects were more profound compared to results presented in a previous study from our lab (Bernardi *et al.* 2015). However, in that study frequencies of T cell populations were reported, whereas numbers are presented herein. In line with effects of E2, the influence of SERMs on thymus cellularity and composition of developing T cells were completely dependent on functional AF-1 and AF-2 of ER α . Overall dependency on both AF-1 and AF-2 in E2-mediated effects on thymus is in accordance with the crucial role for ER α , but not ER β , for mediating E2-induced thymic involution (Lindberg *et al.* 2002).

Bone marrow B cells are reduced during pregnancy, attributed to the high levels of estrogens (Medina & Kincade 1994). We report that the E2-mediated inhibition of total bone marrow CD19⁺ B cell compartment do not require functional AF-1, but is dependent on AF-2. Specifically, E2 suppresses B lymphopoiesis by inhibiting the transition from pro-B to pre-BI stage, and this transition *per se* is

dependent on IL-7 production (von Freeden-Jeffry *et al.* 1995). Herein, we report that the E2-mediated inhibition of pre-BI and immature B cell populations do not require functional AF-1, but is dependent on AF-2. *In vitro* studies have shown that the E2-induced reduction of pre-BI cells is mediated via influence on the IL-7-producing mesenchymal stromal cell compartment rather than by direct effects on the progenitor B cells (which are of hematopoietic origin) (Smithson *et al.* 1995). However, bone marrow chimeric experiments with ER α KO and WT mice showed that E2-mediated reduction in the total B cell frequency (B220⁺ cells) in bone marrow is dependent on ER α expression in hematopoietic cells, although the specific populations of developing B cells were not characterized in that study (Henning *et al.* 2014).

Of note, previously reported effects of E2 that are mediated despite the lack of functional AF-1, are for example increased cortical thickness in the ER α AF-1⁰ mice, whereas effects of E2 on trabecular bone were completely absent in the ER α AF-1⁰ mice (Borjesson *et al.* 2011). Estrogenic effects in cortical bone have been suggested to be mediated via ER α signaling mainly

Figure 6

Roles of functional ER α AF-1 and ER α AF-2 in ER-mediated alterations in late B lymphopoiesis and recirculating mature B cells. OVX ER α AF-1⁰, ER α AF-2⁰ and corresponding littermate wild-type (WT) control mice were treated with 17 β -estradiol (E2: 1 μ g/day), lasofoxifene (LAS: 4 μ g or 8 μ g/day), raloxifene (RAL: 60 or 120 μ g/day) or vehicle for 5 days/week during 3 weeks, with the higher doses of LAS and RAL used in the ER α AF-2⁰ mice and their corresponding WT controls. (A) Representative graph showing gating strategy for assessing immature B cells (B220^{low}IgM⁺) in bone marrow. Frequency of immature B cells in ER α AF-1⁰ mice (B), ER α AF-2⁰ mice (C) and corresponding WT controls. (D) Representative graph showing gating strategy for assessing mature B cells in bone marrow (B220^{high}CD93⁻). Frequency of mature B cells in ER α AF-1⁰ mice (E), ER α AF-2⁰ mice (F) and corresponding WT controls. All populations are expressed as frequency of parent lymphocyte gate. Statistical differences between vehicle and treatment groups were analyzed within each genotype, with ANOVA, except from data in (B: KO; E: WT) which were analyzed with ANCOVA (termination day as covariate). Dunnett's *t* post hoc test was used except when unequal variances were found as in (C: WT), then Dunnett's T3 post hoc test was used. Mean $\pm$ s.e.m. is shown, $n=4-10$ mice/group. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs vehicle.

in osteoblasts, whereas effects on trabecular bone is mediated via ER α in osteoclasts, albeit there are studies showing that ER α signaling in osteoblasts are important for estrogenic effects in trabecular bone as well (Nakamura *et al.* 2007, Almeida *et al.* 2013, Melville *et al.* 2014). Besides regulating bone formation, osteoblasts can also regulate early B lymphopoiesis by producing IL-7 (Zhu *et al.* 2007). Further studies on the role of ER α AF-1 in IL-7-producing cells residing in bone marrow, could possibly aid in understanding the independency of ER α AF-1 for estrogenic response in cortical bone and in the pre-BI cell population.

Reductions in total bone marrow cellularities after SERM treatments were, as for E2, completely dependent on both functional AF-1 and AF-2. Detailed characterization of the developing bone marrow B cells revealed divergent properties of RAL and LAS in terms of necessity of functional AF-1 and AF-2 for altering B lymphopoiesis. Both SERMs reduced pre-BI cells, and the effect of RAL

was only partly dependent on both AF-1 and AF-2, in contrast to the effect of LAS, which was completely dependent on the presence of AF-1, but not AF-2. Since the AF-1 and AF-2 domains are crucial for recruiting transcriptional coregulators to the ER in order to mediate genomic effects, the AF-1/2-independent effects of RAL on pre-BI cells can possibly be mediated by non-genomic ER α signaling instead, such as rapid membrane-initiated steroid signals (MISS). Another possible explanation for the partly ER α AF-1/2-independent effects of RAL on pre-BI cells is signaling via ER β , which is in accordance with our previous studies demonstrating the requirement of functional ER β to achieve full estrogenic response in the regulation of B lymphopoiesis (Erlandsson *et al.* 2003). Furthermore, as discussed previously in the context of E2 effects, IL-7 is a key cytokine in the transition from pro-B cells to pre-B cells, and the impact of SERM on IL-7 has not been studied but could be of importance in understanding the herein reported effects.

RAL mediated a remarkable increase in mature B cells in bone marrow, which has not been reported elsewhere, and for this effect AF-2, but not AF-1, was required. Previous *in vivo* and *in vitro* studies have shown that effects of SERMs are in general dependent on the expression of functional ER α AF-1 (Berry *et al.* 1990, Borjesson *et al.* 2016). This notion is questioned in our study, where we found that effects of raloxifene on B lymphopoiesis (i.e. in pre-BI and mature B cell populations) were only slightly hampered in the *ER α AF-1⁰* mice. These findings could be of clinical importance since humans express the naturally occurring AF-1-deficient truncated 46kDa ER α protein (lacking aa 1–173), which is present in as much as 70% of ER-positive breast cancer cases (Chantalat *et al.* 2016). The SERMs raloxifene and tamoxifene are utilized as prevention and treatment for ER-positive breast cancer due to their ER antagonistic actions in mammary tissue.

One drawback of this study in terms of functional AF-1 vs AF-2 of the ER α in the immunological effects of SERM is the use of different doses of SERM in *ER α AF-1⁰* vs *ER α AF-2⁰* experiments. The *ER α AF-1⁰* experiment was performed several months before *ER α AF-2⁰* experiment, and results on bone analyses in the *ER α AF-1⁰* experiment raised concerns about effective doses of SERM in mice (Borjesson *et al.* 2016). For example, LAS used at 4 μ g/day did not significantly increase BV/TV when analyzed with DEXA, whereas BV/TV was increased in μ CT analysis (Borjesson *et al.* 2016). The opposite was reported for RAL used at 60 μ g/day, in the same study. Thus, doses of SERM were doubled in the *ER α AF-2⁰* experiment to ensure a robust consistent effect on bone—the primary pharmacologic target of these SERMs. The *ER α AF-2⁰* mice and their littermate WT controls received doses of RAL and LAS twice as high compared with *ER α AF-1⁰* mice and corresponding WT, although from a physiological point of view, is not a substantial difference. In general, the effects of SERM on the different immune parameters are similar between the WT mice in the different experiments; nevertheless, in some cases, a dose effect of SERM could possibly explain the slightly more profound effects of treatment with SERM in the WT controls of the AF-2 experiment compared with the effects of SERM treatment in the AF-1 controls. Such examples are found in the SERM-mediated effects on number of thymic DP cells, BM cellularity and frequency of pro-B cells; therefore, the importance of AF-1 in some of these SERM-mediated effects is uncertain.

The profound effects of E2 on T lymphopoiesis in thymus were strictly dependent on both functional AF-1 and AF-2 of ER α . Moreover, both E2 and SERMs alter

developmental stages of B cells in bone marrow, and interestingly, SERM-mediated alteration of B lymphopoiesis showed stage- and substance-specific independence of ER α AF-1 and/or ER α AF-2 revealing divergent roles of AF-1 and AF-2 in E2- and SERM-mediated modulation of B lymphopoiesis. The substance-specific roles of AF-1 and AF-2 of the ER α are new clues in the understanding of the molecular actions of SERMs.

Declaration of interest

H C received lasofoxifene from Pfizer, which approved the final version of the manuscript but did not interfere with the study in any other matter. The other authors have nothing to declare.

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