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Estradiol Promotes Functional Responses in Inflammatory and Steady-State Dendritic Cells through Differential Requirement for Activation Function-1 of Estrogen Receptor α

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17β-Estradiol (E2) has been shown to regulate GM-CSF– or Flt3 ligand–driven dendritic cell (DC) development through estrogen receptor (ER) α signaling in myeloid progenitors. ERs regulates transcription of target genes through two distinct activation functions (AFs), AF-1 and AF-2, whose respective involvement varies in a cell type– or tissue-specific manner. In this study, we investigated the role of ERα AFs in the development and effector functions of inflammatory DCs, steady-state conventional DCs, and plasmacytoid DCs (pDC), using mouse lacking either AF-1 or AF-2. In agreement with previous works, we showed that E2 fostered the differentiation and effector functions of inflammatory DCs through ERα-dependent upregulation of IFN regulatory factor (IRF)-4 in GM-CSF–stimulated myeloid progenitors. Interestingly, whereas AF-1 was required for early IRF-4 upregulation in DC precursors, it was dispensable to enhance IRF-4 expression in differentiated DCs to a level compatible with the development of the more functional Ly6C⁺/CD11b⁺ DC subset. Presence of E2 had no effect on progenitors from either knock-in mice with 7-aa deletion in helix 12 of ERα, lacking AF-2, or ERα⁻/⁻ mice. By contrast, in Flt3 ligand–driven DC differentiation, activation of AF-1 domain was required to promote the development of more functionally competent conventional DCs and pDCs. Moreover, lack of ERα AF-1 blunted the TLR7-mediated IFN-α response of female pDCs in vivo. Thus, our study demonstrates that ERα uses AF-1 differently in steady-state and inflammatory DC lineages to regulate their innate functions, suggesting that selective ER modulators could be used to target specific DC subsets. The Journal of Immunology, 2013, 190: 5459–5470.

Sex hormones such as estrogens influence autoimmunity and the immune response to infection (1, 2). Estrogen effects are mediated by estrogen receptors (ERs) ERα and ERβ, which function as ligand-induced transcription factors (3). This regulatory action of estrogens on immune responses and autoimmunity are thought to be due to a direct action on immunocompetent cells of the innate and adaptive immune systems (4), which have been recently shown to functionally express ERs, particularly ERα (5–11). Alternatively, estrogens, through their ERs, may act on precursor cells to regulate the differentiation and functions of lymphoid and myeloid cells (12–18).

Dendritic cells (DCs) are professional APCs that bridge innate and adaptive immunity. They are essential for activation of naïve T cells specific for self or nonself protein Ags and for their subsequent differentiation into effector T cells through the secretion of specific cytokines (19). Multiple DC subsets have been identified, which are divided into four main cell types: conventional/classical DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells, and monocyte-derived CD11b⁺ inflammatory/migratory DCs (19, 20). Although cDCs are specialized for Ag processing and presentation at the steady-state, under inflammatory conditions, circulating blood monocytes can be rapidly mobilized and differentiate into cells with many features of cDC (19). By contrast, pDCs are a distinct lineage, characterized by their capability to rapidly produce great amounts of type I IFNs in response to viral infections (21).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AF, activation function; BM, bone marrow; cDC, conventional/classical dendritic cell; CM, conventional medium containing phenol red and regular FCS; DC, dendritic cell; E2, 17β-estradiol; ER, estrogen receptor; ERαAF1, knock-in mice lacking the A/B domain of ERα; ERαAF2, knock-in mice with 7-aa deletion in helix 12 of ERα; Flt3L, Flt3L-derived dendritic cell; Flt3L, Flt3 ligand; GM-DC, GM-CSF–derived dendritic cell; GMFI, geometric mean fluorescence intensity; IRF, IFN regulatory factor; MHC-II, MHC class II; MISS, membrane-initiated steroid signaling; MP, myeloid progenitor; pDC, plasmacytoid dendritic cell; SFM, steroid-free medium; WT, wild-type.

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Several studies have shown a role for ERα signaling in the differentiation and functions of various DC subsets not only in vitro (13, 16–18), but also in vivo (11). DCs can be generated from bone marrow (BM) precursors cultured in the presence of GM-CSF or Flt3 ligand (Flt3L). GM-CSF induces mainly myeloid DCs (GM-CSF–derived DC [GM-DC]) thought to be equivalent to monocyte-derived CD11b+ DCs, whereas Flt3L allows the development of DCs (Flt3L-derived DCs [FL-DCs]), similar in phenotype and function to splenic resident cDC and pDCs (19, 20, 22). Agonists or antagonists of ERα can differentially regulate the development of these distinct DC populations in vitro (13, 16, 17, 23, 24). For instance, 17β-estradiol (E2) is crucial for the GM-CSF–dependent differentiation of DCs (13, 17), through ERα but not ERβ (17). Interestingly, Lin− c-kit+ Flt3+ myeloid progenitors (MPs) express high levels of ERα but not ERβ (16), and respond to E2 by up-regulating IFN regulatory factor (IRF)-4 in the presence of GM-CSF, thereby promoting DC differentiation (18). Besides differentiation, E2 augments the innate function and T cell–stimulatory activity of GM-DCs (17). Although E2 effects on FL-DC innate function have not previously been examined, we reported recently that cell-intrinsic ERα signaling by estradiol positively regulated the endosomal TLR-mediated responses of human and mouse pDCs in vivo (11). Altogether, these results support a critical regulatory role for ERα activation in both development and innate function of all DC subsets.

Because selective ER modulators like tamoxifen and raloxifene are in current clinical use, it is important to decipher the mechanisms by which ERs signaling regulates inflammatory and homeostatic DC development and functions. Full-length ERα (66 kDa) consists of six domains (A–F) and two separate transactivation functions, activation function (AF)-1 and AF-2, which reside in the N-terminal A/B domain and the C-terminal E domain, respectively (25, 26). A truncated isoform of ERα (46 kDa) lacking the terminal A/B domain and therefore devoid of AF-1 has been shown to be expressed in various tissues and to mediate E2-dependent transcriptional responses (27, 28). Full ligand-dependent ERα activation entails interactions between the AF domains, but AF-1 and AF-2 can activate transcription independently in a promoter- and cell type–specific manner (26, 29, 30). It has been proposed that the relative contribution of AF-1 and AF-2 in ERα transcriptional activity depends on the differentiation stage of the cells (31) or the tissue examined (32). Mice lacking either AF-1 (knock-in mice lacking the A/B domain of ERα [ERαAF1⁻]) or AF-2 (knock-in mice with 7-aa deletion in helix 12 of ERα [ERαAF2⁻]) have been recently generated (33–35). It has been shown using these models that ERαAF1⁻ was dispensable for E2-induced vascular protection (33) and osteoporosis prevention (35). By contrast, AF-1 is necessary for the proliferation of breast cancer cells (36) and for the normal E2-induced increase in uterine weight in vivo (33). Unlike AF-1, AF-2 was required for all E2-mediated actions with the exception of acceleration of endothelial healing (34, 35). These results indicate that the requirement for AF-1 is largely tissue dependent.

In this study, we have evaluated the effect of E2 on the development and functional properties of GM-DCs and FL-DCs using recently described mouse lines deficient for either AF-1 or AF-2 ERα activity (33, 34), aiming to dissect the differential regulation by ERα AFs of inflammatory versus homeostatic DCs.

Materials and Methods
Mice
C57BL/6 (B6) (H-2b, CD45.2) mice were purchased from Centre d’Elevage R. Janvier. ERα-deficient (ERα−/−), ERαF1⁻, and ERαF2⁻ mice on C57BL/6 background have been previously described (33, 34, 37). ERαF1⁻ has been generated by targeting the first exon of ERα gene (aa 2–148) coding the A and B domains (33). These mice expressed normal level of a truncated ERα protein that lacks the A domain and all three motifs contributing to ERα AF-1 in the B domain. ERαF2⁻ mice were generated by deleting 7a aa in the helix 12 corresponding to aa 543–549 and were devoid of AF-2 (34). CD45.1 B6.SJL congenic mice were initially obtained from The Jackson Laboratory. Mice were bred and maintained in our specific pathogen-free animal facility. Protocols were approved by our institutional review board for animal experimentation. Irradiation BM chimeras were generated as described previously (11). Unless otherwise stated, most experiments were performed with male cells, but similar results were obtained with female cells. Mice were matched for sex in all experiments.

Cell culture reagents
Conventional medium (referred as CM) was RPMI 1640 supplemented with 10% heat-inactivated FCS (Lonza). 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM l-glutamine, 50 μM 2-ME, and 50 μg/ml gentamicin (Sigma). In estrogen-controlled condition (referred as steroid-free medium [SFM]) experiments, phenol red–free RPMI 1640 with 10% dextran charcoal-treated FCS (HyClone) was used.

DC generation from murine BM
BM-derived DCs were generated in conventional medium (CM) or SFM supplemented with murine GM-CSF as previously described (17). In Flt3L-driven model (FL-DC), BM was seeded at 2.10⁶ cells/ml supplemented with murine Flt3L (200 ng/ml) in SFM. Recombinant murine Flt3L was produced in-house in insect cells as described previously (38). Both GM-DCs and FL-DCs were harvested and analyzed on days 8–9. Culture were supplemented or not with E2 (Sigma) dissolved in DMSO (10⁻⁴ M) and added at a final concentration of 10⁻² to 10⁻⁴ M. Viable cell numbers for each culture were determined by trypan blue exclusion. Cell counts were used to calculate the absolute numbers of each DC subset according to the flow cytometry analysis.

Cell sorting of MPs and quantitative PCR analysis
A first enrichment step was done using rat Abs against TER-119, CD19, CD11b, and CD3 and anti–rat-coated Dynabeads (Dynal), to isolate MPs. Negatively selected cells were then labeled with FITC-labeled mAb directed at the lineage-specific markers (CD3, CD19, NK1.1, Ter119, B220, CD11c, CD11b, Gr1 [Ly6-G/Ly-6C], Sca-1, c-kit–PECy7, and Flt3–allophycocyanin (all from BD Biosciences). MP cells were identified as Lin⁺ Sca-1⁻ c-kit⁻ Flt3⁺ cells as described elsewhere (39) and sorted on a FACSaria (BD Biosciences). MP purity was routinely >98% after postsorts analyses. MP (10–15 × 10⁶ cells/well) were cultured in SFM supplemented with GM-CSF in the presence or not of 2.10⁻³ to 10⁻⁴ M for indicated times. Total RNA was extracted (NanoPrep kit; Agilent Technologies) and reverse transcribed with oligo-dT, random hexamers, and SuperScript RT III (Invitrogen). Semi-quantitative RT-PCR was performed using SybrGreen PCR Master Mix (Roche). Gene transcripts were normalized to B-actin gene abundance, and relative mRNA levels were calculated by the expression 2⁻DDCT. The following primers were used: hB-f, forward 5′-CTATCCCACTACAAGCCTCCT-3′; hB-r, reverse 5′-TGTACAACTGGGACGAC-3′; hB-actin, forward 5′-TGGATGATTGCAGCCACCTCG-3′; hB-actin, reverse 5′-CTGGGGCATCTTTTACCGGT-3′. IRF4–specific primers have been previously described (40).

DC purification
DCs were purified from GM-CSF and Flt3L cultures using CD11c-specific microbeads (Miltenyi). For Flt3L culture, a depletion of CD45RA+ cells by unconjugated anti-CD45RA (BioLegend) followed by anti-rat–coated Dynabeads (Dynal) was performed. BM-derived DCs were generated in conventional medium (CM) or SFM supplemented with murine GM-CSF as previously described (17). In Flt3L-driven model (FL-DC), BM was seeded at 2.10⁶ cells/ml supplemented with murine Flt3L (200 ng/ml) in SFM. Recombinant murine Flt3L was produced in-house in insect cells as described previously (38). Both GM-DCs and FL-DCs were harvested and analyzed on days 8–9. Culture were supplemented or not with E2 (Sigma) dissolved in DMSO (10⁻⁴ M) and added at a final concentration of 10⁻² to 10⁻⁴ M. Viable cell numbers for each culture were determined by trypan blue exclusion. Cell counts were used to calculate the absolute numbers of each DC subset according to the flow cytometry analysis.

Flow cytometry
Before staining, cells (5–10 × 10⁶) were incubated 15 min at room temperature with blocking buffer (PBS with 1% FCS, 3% normal mouse serum, 3% normal rat serum, 5 mM EDTA, 1% NaN₃) containing 5 μg/ml anti-CD16/CD32 (2.4G2; American Type Culture Collection). For surface cell staining, cells were incubated for 30 min on ice with FITC-, PE-, biotinylated mAbs were used, cells were washed twice in FACS buffer before incubation with PerCP-Cy5.5–conjugated streptavidin (eBioscience, San Diego, CA). Cells were stained with Abs from BD Bio-
sciences; CD11c (HL3), CD11b (M1/70), Ly6C (AL-21), CD86 (GL1), TLR4 (MT5510), c-kit (2B8), Flt3 (A2F10.1), or Abs from eBioscience; CD11c (N418), anti–MHC class II (anti–MHC-II M5/114.15.2), CD40 (HM40-3), CD45.1 (A20), CD45.2 (104), B220 (RA3-6B2), Sca-1 (D7), CD115 (AF598), PDCA-1 (eBio927), and Siglec-H (eBio440c). Flow cytometry was performed on a FACSCalibur or LSR II flow cytometer (BD Biosciences).

For detection of intracellular cytokine production, purified DCs were stimulated with TLR ligands for 16–18 h and incubated with brefeldin A (eBioscience) for 4 h in the presence of brefeldin A for the last 2 h. After surface staining and fixation in PBS/1% paraformaldehyde, cells were permeabilized with 0.5% saponin and intracellular cytokine staining was performed with Abs specific for IL-6 (MP5-20F3), IL-12p40/p70 (C15.6), IFN-α/β (RMMA-1/RMMB-1; PBL IFNsource), or rat IgG1 isotype control (BD Biosciences). For detection of intracellular IRF-4 and E47, cells were cell-surface stained as described earlier, then fixed and permeabilized using the intracellular fixation/permeabilization buffer set from eBioscience. eFluor 660–conjugated anti–IRF-4 Ab (3E4) and control isotype (rat IgG1, κ) were from eBioscience.

Functional analysis of DCs and ELISAs

Purified DCs were stimulated with indicated amounts of LPS (E. coli 0111:B4 LPS Ultra-Pure), resiquimod R-848, or CpG-1668 (CpG-B) from Invivogen. For CD40-dependent stimulation, purified DCs were cocultured with control mock-transfected or CD40L (CD154)-expressing NIH 3T3 fibroblasts (2.5 × 10^5 cells/well) as previously described (17). Cytokine production in 24-h culture supernatants was quantified by two-site sandwich ELISA as described previously (17).

T cell proliferation

Indicated numbers of enriched DCs from either wild-type (WT), ERα, or EROS/AF1^−/− culture were incubated with magnetically sorted CD4^+ T cells (2.10^5 cells/well) from BALB/c mice. CD4^+ T cell proliferation was assessed by [3H]thymidine incorporation. Cell cultures were pulsed for 10 h with 1 μCi (37 KBq) [3H]thymidine (40 Ci/nmol; Radiochemical Center, GE Healthcare, Little Chalfont, U.K.). Incorporation of [3H]TdR was measured using a MicroBeta TriLux luminescence counter (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Statistical analysis

The statistical significance was estimated with the two-tailed Mann–Whitney U test or one-way ANOVA. A p value ≤0.05 was considered significant in all cases.

Results

ERα signaling is required to promote GM-DC differentiation through an AF-1-independent mechanism

We first evaluated the role of activation of ERα AF-1 on GM-DC development and function using a recently described EROS/AF1^−/− knock-in mouse model in which exon 1 was deleted (33), and we compared it with complete ERα-deficient mice. In agreement with previous works (17), we showed that the frequency, as well as the absolute numbers, of DCs that developed from ERα−/− mice was reduced by 30% compared to WT BM (Fig. 1A, 1B). DCs that developed in absence of ERα activation were enriched in cells expressing high levels of CD11b, low levels of MHC-II, and high level of Ly6C and TLR4 (Fig. 1C, 1D, Supplemental Fig. 1). Additional of E2 enhanced the numbers of conventional CD11b^+ Ly6C^neg GM-DCs (Fig. 1C, 1D), expressing intermediate to high level of MHC-II and lower level of TLR4 (Supplemental Fig. 1), in agreement with previous works (17, 41). Interestingly, whereas E2 had no effect on GM-DC cultures from ERα^−/− mice, it was able to restore GM-DC yield and phenotype in EROS/AF1^−/− BM cultures to levels comparable with those observed in WT cultures (Fig. 1, Supplemental Fig. 1). Although the restoration of the GM-DC phenotypes was not complete in BM cells that lack EROS/AF1, there was a clear and significant response to E2 stimulation (Fig. 1, Supplemental Fig. 1). By contrast, GM-DC cultures from EROS/AF2^−/− mice were totally unresponsive to E2 supplementation, like ERα-deficient BM cells (Supplemental Fig. 2). Thus, AF-2 in ERα is strictly required to promote normal GM-DC development, whereas AF-1 is, in part, dispensable.

Irfl-4 induction in GM-CSF–stimulated lin^− Flt3^+ MPs is controlled by ERα activation through both AF-1 and AF-2

It has been recently shown that E2 strongly enhances Irfl-4 expression in lin^− Flt3^+ MPs stimulated with GM-CSF, thereby
promoting GM-DC development (18). To assess the role of ERα signaling on *Irf4* mRNA expression, we cultured highly purified Lin<sup>−</sup> Sca-1<sup>−</sup> c-kith<sup>−</sup> Flt3<sup>−</sup> MPs (Supplemental Fig. 3) from WT or ERα mutant BM cells with GM-CSF in SFM supplemented or not with E2. The frequencies and absolute numbers of Lin<sup>−</sup> c-kith<sup>−</sup> Flt3<sup>−</sup> CD115<sup>+</sup> common DC precursors, were not reduced in the BM of ERα mutant mice as compared with WT mice (Supplemental Fig. 3). These data indicated that ERα signaling did not impair MP/common DC precursor development in the steady-state. In steroid-free conditions, *Irf-4* was upregulated by 6- to 7-fold after 48 h of stimulation with GM-CSF, and no difference was observed between WT, ERα<sup>−/−</sup>, and ERαAF mutant MPs (Fig. 2A). Thus, in the absence of E2, the GM-CSF–driven induction of *Irf-4* in MPs was not affected by ERα deficiency. In striking contrast, in the presence of E2 (at 10<sup>−9</sup> and 10<sup>−8</sup> M), a marked upregulation of *Irf4* (15-fold increase as compared with t0) was observed in ERα<sup>+/+</sup> MPs, but not in MP cultures from all ERα mutant mice, including ERαAF<sup>+</sup> (Fig. 2A). Similar results were obtained at later time points by 72–96 h (Fig. 2B). As previously reported (18), the expression of other transcription factors, such as PU-1 and Id2, was not significantly affected (<2-fold) by E2 in WT MPs, as well as in ERα<sup>−/−</sup> and ERαAF mutant MPs (data not shown). In agreement with *Irf-4* transcript levels, intracellular IRF-4 protein expression was significantly increased in GM-CSF–stimulated WT MPs exposed to E2 at 48 and 96 h (Fig. 2C). Again, ERαAF1<sup>−/−</sup> MPs, like ERα<sup>−/−</sup> MPs, were unable to upregulate IRF-4 at levels similar to WT MPs at 48 h (Fig. 2C). We noticed, however, that IRF-4 expression level was significantly higher in ERαAF1<sup>−/−</sup> MPs as compared with ERα<sup>−/−</sup> at 96 h (Fig. 2C). Thus, these data indicated that both ERα AF-1 and AF-2 were required to sustain the GM-CSF–dependent induction of IRF-4 in MPs at early stage of differentiation.

**ERα AF-1 is dispensable for the E2-mediated upregulation of effector functions and IRF-4 expression in fully differentiated CD11c<sup>+</sup> DCs**

Interestingly, despite the lack of E2-induced upregulation of IRF-4 in GM-CSF–stimulated MPs at early time points, we observed a restoration of DC yield and phenotype when ERαAF-1<sup>−/−</sup> precursors were grown in the presence of E2 for 7–9 d (Fig. 1, Supplemental Fig. 1). ERα<sup>−/−</sup> and ERαAF1<sup>−/−</sup> DCs were mainly composed of CD11b<sup>+</sup> Ly6C<sup>−</sup> DCs, which have been shown to express higher levels of IRF-4 than ERα<sup>−/−</sup> DCs enriched in the CD11b<sup>+</sup> Ly6C<sup>+</sup> subset (18). We therefore evaluated IRF-4 protein expression in fully differentiated CD11c<sup>+</sup> DCs on day 7. Inter-

**FIGURE 2.** Role of ERα AF-1 and AF-2 on *Irf4* gene expression in Lin<sup>−</sup> Flt3<sup>−</sup> MPs stimulated with GM-CSF. MPs (Lin<sup>−</sup>, Sca-1<sup>−</sup> c-kith<sup>−</sup> Flt3<sup>−</sup>) were FACS sorted from WT, ERα<sup>−/−</sup>, ERαAF1<sup>−/−</sup>, and ERαAF2<sup>−/−</sup> mice. (A) Purified MPs (10–15 × 10<sup>3</sup> cells/well) were stimulated with GM-CSF for 48 h in SFM supplemented with 10<sup>−9</sup> M E2. In (B), sorted MPs from WT, ERα<sup>−/−</sup>, and ERαAF1<sup>−/−</sup> mice were stimulated with GM-CSF in E2-supplemented medium (10<sup>−9</sup> M) for 72 and 96 h. The expression of transcripts coding for the *Irf4* was determined by real-time RT-PCR and normalized to β-actin gene expression. (C) Sorted MPs were stimulated with GM-CSF in SFM supplemented with 10<sup>−9</sup> M E2. Expression of *Irf-4* was then determined at 48 and 96 h by flow cytometry. Histograms represent the geometric mean fluorescence intensity (GMFI) of IRF-4. Results from replicate cultures (n = 3–4) are shown and are from one representative experiment from two to four performed with similar results. Error bars represent SD. (D) BM cells from WT, ERα<sup>−/−</sup>, ERαAF1<sup>−/−</sup>, and ERαAF2<sup>−/−</sup> mice were grown with GM-CSF in SFM supplemented or not with 10<sup>−9</sup> M E2. At day 7 of culture, the GMFI of IRF-4 was determined by flow cytometry. Histograms represent the GMFI fold change relative to WT BM cells without E2. Results from replicate cultures (n = 4) are shown and are from one representative experiment from two performed with similar results. Error bars represent SD. **p < 0.01, ***p < 0.001, one-way ANOVA.
estingly, the enhanced differentiation of Ly6C<sup>−</sup> CD11b<sup>+</sup> subset in the presence of E2 in both WT and ERαAF1<sup>−/−</sup> DC cultures (Fig. 1) was correlated with a ligand-dependent upregulation of IRF-4 protein in WT and ERαAF1<sup>−/−</sup> CD11c<sup>+</sup> cells (Fig. 2D), although IRF-4 expression was still significantly lower in ERαAF1<sup>−/−</sup> DCs as compared with WT DCs (Fig. 2D). By contrast, E2 exposure had no effect on IRF-4 expression in ERα<sup>−/−</sup>- and ERαAF2<sup>−/−</sup> DCs (Fig. 2D).

Because ERα signaling has been shown to promote the development of Ly6C<sup>−</sup> CD11b<sup>+</sup> DCs with enhanced functional properties (17), we next evaluated whether E2 could enhance the proinflammatory functions of ERαAF1<sup>−/−</sup> DC as efficiently as in WT DCs (17). As shown in Fig. 3A, both WT and ERαAF1<sup>−/−</sup> CD11c<sup>+</sup> DCs generated in the presence of E2 produced comparable levels of IL-6 and IL12p40 upon stimulation with CpG. By contrast, the TLR9-mediated production of cytokines was reduced in CD11c<sup>+</sup> DCs generated in the absence of E2 (Fig. 3A), whether cells were generated from ERαAF1<sup>−/−</sup> or WT BM cells (data not shown). A similar tendency was found when we examined the cytokine response of DCs upon CD40L stimulation (Fig. 3B). Both WT and ERαAF1<sup>−/−</sup> CD11c<sup>+</sup> DCs generated in E2-supplemented medium had an enhanced capacity to produce IL-6, as compared with ERα<sup>−/−</sup> CD11c<sup>+</sup> DCs, upon CD40 engagement using CD40L-expressing fibroblasts (Fig. 3B).

We next evaluated the ability of these DC populations to prime an alloreactive MLR. Purified CD11c<sup>+</sup> DCs were generated from ERα<sup>+/+</sup>, ERα<sup>−/−</sup>, or ERαAF1<sup>−/−</sup> mice in the presence of E2 and then used to stimulate alloreactive CD4<sup>+</sup> T cells from BALB/c mice. Again, WT and ERαAF1<sup>−/−</sup> CD11c<sup>+</sup> DCs were equally efficient in inducing the proliferation of BALB/c CD4<sup>+</sup> T cells and were both superior to ERα<sup>−/−</sup> DCs (Fig. 3C). Together, these data further showed that ERα signaling was required to promote the development of inflammatory DCs with optimal innate and adaptive immune functions, and that ERα AF-1 was dispensable for most of these effects of E2.

**ERαAF1 is required for the E2-mediated actions on Flt3L-induced cDC and pDC development**

We next evaluated the effect of E2 on Flt3L-driven cDC and pDC development. A previous study showed that E2 diminished the yield of DCs, but the impact of E2 on cDC and pDC innate functions was not previously examined (16). Analysis of WT subpopulations showed that pDC (CD11c<sup>+</sup>B220<sup>−</sup>, fold reduction 1.82), rather than cDC (CD11c<sup>+</sup>B220<sup>−</sup>Flt3L<sup>+</sup>, fold reduction 1.4), absolute numbers were mainly affected by ERα signaling during their development. By contrast, none of the DC populations derived from ERα<sup>−/−</sup> or ERαAF1<sup>−/−</sup> BM were modified by the addition of E2 during the culture period (Fig. 4A, 4B). These results show that ERα signaling negatively regulates the absolute numbers of FL-DC in vitro, mainly affecting the pDC subset, through a mechanism that requires the ERα AF-1.

Because E2 modulated GM-DC maturation, we then assessed whether FL-cDCs that developed in the presence of E2 exhibited different expression of MHC-II and costimulatory molecules, required to mediate cDC adaptive function. The percentage of CD11c<sup>+</sup>B220<sup>+</sup> cDCs was increased in WT DC cultures in the presence of E2, as compared with those generated from ERα<sup>−/−</sup> or ERαAF1<sup>−/−</sup> mutant mice (Fig. 4A). Furthermore, cDCs that developed from ERα<sup>−/−</sup> or ERαAF1<sup>−/−</sup> BM cells exhibited a less mature phenotype, with an increased proportion of DCs expressing low levels of MHC-II (Fig. 4C). Although ERα activation slightly upregulated (±10%) the basal expression of CD40 or CD86 molecules on WT cDCs (Supplemental Fig. 4), we observed a 50% increase in the expression of CD40 and CD86 molecules on pDCs from WT cells generated in the presence of E2 (Supplemental Fig. 4). As shown in Fig. 4C, the frequency of pDCs expressing high levels of MHC-II molecules was increased by 15–20% in E2-supplemented cultures. By contrast, E2 supplementation had no effect on both MHC-II CD40 or CD86 expression on pDCs when BM cells were obtained from ERα<sup>−/−</sup> or ERαAF1<sup>−/−</sup> mice. Thus, E2 acts in the presence of Flt3L to promote the emergence of more mature cDC and pDC populations. Interestingly, unlike inflammatory DCs that developed in the presence of GM-CSF, these effects of E2 required the ERαAF1 activity.

**E2 positively regulates the TLR-mediated responses of cDCs through ERαAF1**

We next assessed whether ligand-inducible ERα activation during FL-DC development could also modulate the innate function of CD11c<sup>+</sup>B220<sup>−</sup> cDCs. The analysis of MHC-II and CD40L expression after TLR4 stimulation showed that the proportion of mature DCs, MHC-II<sup>high</sup> or CD40<sup>+/−</sup>CD86<sup>high</sup>, was substantially increased when FL-DCs were differentiated in the presence of E2. This effect of E2 was lost in cDCs generated from both ERα<sup>−/−</sup> or
ERαAF-1° mice that resembled WT cDCs generated in the absence of E2 (Fig. 5A). We then tested the capacity of purified cDCs, derived from WT, ERα−/−, or ERαAF1° were grown for 9 d with Flt3L in SFM with DMSO or 10−9 M E2. (A) The percentages of cDCs (CD11c+ B220+) and pDCs (CD11c− B220+) in the cultures were determined by flow cytometry and are indicated in the dot plots. (B) Absolute numbers of CD11c+ DC, cDC, and pDC subsets generated in the presence (black bar) or absence (white bar) of E2. Data are combined results from seven independent experiments (mean ± SEM). (C and D) MHC-II expression on cDCs (upper panels) and pDCs (lower panels) was analyzed by flow cytometry. Percentage of MHC-II-expressing cells is indicated. (D) Bar graphs show the percentage of MHC-IIhigh cDCs and pDCs from triplicate culture (mean ± SEM). Results are representative of four independent experiments. *p < 0.05, **p < 0.01.

Cell-intrinsic ERα signaling controls the phenotypic changes and the enhanced TLR-mediated responses of cDCs

To investigate whether the E2 effect on FL-cDCs was caused by a cell-intrinsic defect of ERα signaling, we cocultured either WT or ERα−/− BM cells with equal numbers of Ly-5.1 WT BM and analyzed the cDC phenotype and function after 9 d of Flt3L-mediated differentiation in the presence of E2. In agreement with data in Fig. 4, the frequency of cDCs was increased in DC cultures developing from WT progenitors, representing 40% of total cells (Fig. 6A). We did not observe a competitive advantage conferred by ERα deficiency for cDC development (Fig. 6A), supporting our results showing that E2 effect on DC yield mainly affects pDCs (Fig. 4). In the presence of E2, we observed an enrichment of cDCs expressing high levels of MHC-II molecules in CD45.1° WT but not in ERα−/− CD45.2° cells (Fig. 6B), indicating that cell-intrinsic ERα signaling rather than soluble factors regulate MHC-II expression on cDC populations.

We next sorted CD45.1 WT and CD45.2 WT or ERα−/− cDCs from these cocultures and assessed their functional properties. As shown in Fig. 6C, WT DCs differentiated in the presence of E2 secreted large amounts of IL-6 and IL12p70 after TLR4/CD40L stimulation. In contrast, cytokine levels produced by cDCs that developed in absence of E2, or from ERα−/− progenitors, were strongly reduced compared with WT DC production. WT CD45.1 cDCs that developed in the presence of CD45.2 ERα−/− or CD45.2 WT cells produced the same amounts of IL-6 or IL-12 (Fig. 6C). These results demonstrate that E2 acts through ERα, in a cell-intrinsic manner, to promote cDC effector functions.

The presence of E2 during Flt3L-induced pDC differentiation enhances their TLR7- and TLR9-mediated responses through ERα AF-1 signaling

We recently demonstrated that E2 enhanced pDC innate functions in vivo in both humans and mice (11). We therefore examined the
TLR-mediated responses of purified pDCs generated from WT, ERα−/−, or ERαAF1−/− BM cells. (A) DCs were stimulated with LPS (1 μg/ml) overnight, and MHC-II, CD40, and CD86 expression were analyzed by flow cytometry. Shaded histograms represent DCs cultured in medium alone and bold lines those stimulated with LPS. (B) Purified cDC generated as in (A) were cultured on a monolayer of CD40L-expressing fibroblasts and stimulated with LPS. After 12 h, IL-12p40- and IL-6-expressing CD11c+ DCs were analyzed by intracellular staining. (C) Bar graphs show the relative change of cytokine-expressing DCs derived in the presence of E2 compared with E2-deprived cultures. (D) Purified cDCs were stimulated as in (B), and IL-12p70 and IL-6 were measured by ELISA in 24-h culture supernatants. Bar graphs show fold change in cytokine concentration between cDCs generated in SFM supplemented or not with E2. Data are combined results from three independent experiments (mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.
these results demonstrate the crucial role of ERα AF-1 in the ability of endogenous estrogens to enhance the TLR responses of female pDCs in vivo.

**Discussion**

In this work, we investigated whether discrete AF domains of ERα could differentially regulate the development and functional properties of the DC subsets that develop in the presence of GM-CSF or Flt3L. These cytokine-driven differentiation pathways are thought to account for the main DC subsets that develop during inflammatory or steady-state conditions, respectively (19, 20, 22).

In these two systems, our data show that E2 acts through ERα to promote the development of DCs with a more mature phenotype with enhanced proinflammatory functions. The ligand-induced activation of ERα involves the actions of distinct AFs, which contributes to the ERα activity depending on the cell type and promoter context (26, 29, 30). Indeed, it has been recently shown using AF targeted mice that ERα AF-1 mediated physiological responses in certain tissues but was dispensable in others (33–35, 42). Using similar validated models, we now establish that the E2-mediated effects on GM- or FL-DC development and effector functions are mediated through ERα AF-1–independent or –dependent mechanisms, respectively.

Among the genes potentially regulated by ERα activation in developing DCs, IRF-4 has been recently shown as a potential target of estrogen action (18). The IRF family members IRF-4 and IRF-8 play critical roles in the development and function of DCs (19, 43, 44). Although both factors have been shown to exhibit overlapping activity, they also possess DC subset–specific functions (43). Whereas IRF-8 is required for CD8α+ cDC and pDC development in vivo and for Flt3L-mediated DC differentiation in vitro (45–47), IRF-4 is essential for the GM-CSF–mediated DC...
FIGURE 7. E2 positively regulates the TLR-mediated response of BM-derived pDCs through ERx in an AF-1–dependent mechanism. FL-DCs from WT, ERx–/-, or ERxAF1˚ BM cells were generated in medium containing regular FCS and E2 (10⁻¹⁰ M). At day 9, pDCs were purified by FACS on the basis of CD11c B220 expression and cultured in the presence of CpG-B (A) or R-848 (B). The production of IL-12p40 (A, B) was assessed in 24-h culture supernatants by ELISA. (C and D) pDCs from WT, ERx–/-, or ERxAF1˚ BM cells were generated in SFM supplemented or not with 10⁻⁸ M E2. CD45RA⁺ cells from cultures were purified by magnetic selection. Purified pDCs were stimulated with R848 (3 μg/ml) for 18 h, and IL-12p40-expressing pDCs were analyzed by intracellular staining. (D) Bar graphs show the relative change of cytokine expressing DCs derived in the presence of E2 compared with E2-deprived cultures. *p < 0.05, **p < 0.01. Results are presented as mean ± SEM of a pool of four independent experiments. n.s., No significance.

were mediated by a pool of intracellular receptors localized at the plasma membrane in caveolae rafts and have been mainly described in cultured cells in vitro (50, 51). The 46-kDa ERx protein lacking AF-1 is a natural isoform of ERx, which has been shown to localize preferentially at the plasma membrane and to mediate membrane-initiated steroid signaling (MISS) in endothelial cells (50). Furthermore, we recently showed that E2-induced reendothelialization, the unique MISS effect of estrogens described in vivo to date (52), was totally conserved in ERxAF2˚ mice (34). Altogether, the lack of E2 effect in ERxAF2˚ mice indicates that MISS effects are not sufficient to mediate the E2 actions on GM-DC differentiation.

The observation that AF-1 synergizes with AF-2 to enhance the GM-CSF–dependent expression of IRF-4 in MPs at early stage, whereas it was dispensable at later stage of IRF-4–dependent DC development, suggests indirect mechanisms of IRF-4 regulation by E2/ERx signaling. AF-2 plays a critical role in regulating the interaction of ERx with most of the coregulatory proteins, but AF-1–specific coactivators have also been described (32). ERx might regulate IRF-4 through the recruitment of coactivators (53) and/or the induction of other transcription factors, which may both require synergistic interactions between both AFs. In the absence of ERx AF-1, such rate-limiting factors may necessitate a longer signaling period to achieve threshold levels required to promote IRF-4 expression. This would fit with the delayed upregulation of IRF-4 in ERxAF1˚ MP as compared with ERx-deficient ones, although many other mechanisms could be at play.

Interestingly, in the Flt3L model, we also found that E2 induces the development of more functionally competent cDCs and pDCs. By contrast with GM-DC, we found that most of the E2-mediated effect on FL-DC development and functions was abolished not only in ERx–/-, but also in ERxAF1˚ BM cell cultures. Supporting previous work, we showed that E2 diminished the yield of FL-DCs, particularly pDCs as compared with cDCs that were relatively spared (16). Importantly, we demonstrated that E2, through ERx, enhanced the expression of MHC-II and costimulatory molecules...
on TLR-stimulated cDCs subsets, as well as their capacity to produce IL-12 and IL-6, upon stimulation through both TLR4 and CD40. These data are consistent with a key role of estrogens in promoting the development of DCs with a more mature functional phenotype characterized by enhanced TLR-mediated responses. Indeed, we recently provided evidence that the endosomal TLR7- and TLR9-mediated responses of human and mouse pDCs were positively regulated by estradiol in vivo (11), which may account for the sex-dependent differences in the TLR-mediated response of human pDCs (54, 55). This enhancing effect of estrogens was mediated through cell-intrinsic ERα signaling in CD11c+ cells (11), and we show in this article that this effect was strictly dependent on ERα AF-1. Although this AF-1 requirement may point to a dominant role of genomic actions of ERα signaling on target genes, nongenomic mechanisms could also be at play. Indeed, the PI3K/PKB signaling pathway can be activated by acute exposure to E2 in vitro in different cell types including endothelial cells and cortical neurons through MISS effects (56–58), and enhanced PKB activity has been recently shown to augment human pDC development and function (59). The continuous presence of E2 in culture could therefore promote PI3K-PKB activation in developing pDCs (59), thereby enhancing their capacity to respond to subsequent TLR stimulation. Alternatively, the AF-1 region of ERα contains multiple Ser residues that can be phosphorylated by several kinases including PKB and MAPK (60). ERα phosphorylation has been shown to influence the recruitment of coactivators, resulting in enhanced ERα-mediated transcription (60).

The requirement for AF-1 phosphorylation in the E2-mediated effect on cytokine-driven DC development and innate function remains, however, to be investigated.

In conclusion, our data indicate that E2 through ERα exerts proinflammatory effects on both GM-CSF–derived DCs and FL-DCs, probably by promoting the differentiation of DCs that exhibit superior innate functions. As it has been shown for others tissues (33–35, 42), these effects were mediated through a differential requirement for ERα AF-1 that was DC subset specific. Although our work suggests that the impact of E2 on cytokine-driven DC differentiation mainly results from AF-1–dependent or –independent genomic effects, the contribution of nongenomic MISS actions remains to be addressed. Understanding further the respective contribution of ERα subfunctions on DC biology may help select selective ER modulators able to differentially regulate steady-state resident and inflammatory DCs in vivo, to optimize selective ERα modulation of innate immunity in various pathophysiological contexts.

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Disclosures
The authors have no financial conflicts of interest.


Figure S1: AF-1-independent activation of ERα is sufficient to partially restore the phenotypic changes induced by E2 on DC-GM. BM cells from WT, ERα−/− or ERαAF10 were grown for 9 days with GM-CSF in steroid-free medium (SFM) supplemented or not with E2 10⁻⁹ M (SFM+E2). (A-C) Phenotypic changes induced by E2 were assessed by flow cytometry on WT (A), ERα−/− (B) and AF1−/− (C). The percentages of CD11b<sup>high</sup> TLR-4⁺, CD11b<sup>low</sup> MHC-II⁺, TLR4<sup>low</sup> MHC-II⁺ among CD11c⁺ DCs are indicated. Data are representative of four independent experiments.
Figure S2: ERα AF2 is required to promote E2 responsiveness in DC-GM cultures. BM cells from WT or ERαAF20 (AF20) were grown with GM-CSF in steroid-free medium (SFM) supplemented with E2. (A-C) Phenotypic changes induced by E2 were assessed by flow cytometry at day 8 of culture. The percentages (A and B) and absolute numbers (C) of CD11c+ cells are shown from triplicate cultures. In panel D, the percentages of CD11b\textsuperscript{low} MHC-II+ cells among CD11c+ DCs are indicated. Data are representative of two independent experiments.
Figure S3: Gating strategy for MP cell sorting and progenitor analysis in ERα mutant mice. (A) Lineage-depleted BM cells were stained for lineage marker (Lin), c-kit, Sca-1 and Flt3 as described in Methods. MPs were then sorted as Lin^- c-Kit^ Sca-1^{neg} Flt3^+ cells. The gating procedure used for isolation of MP and the post-sort analysis are shown. (B) Gating procedure used to analyse MP (Lin^- c-Kit^{high} Flt3^+) and CDP (Lin^- c-Kit^{int} Flt3^+ CD115^+) cell frequency in total BM cells. Frequency and total number of MP (C) and CDP (D) cells from WT, ERα^-/-, ERαAF1^0 and ERαAF2^0 mice. Ten to 14 week-old female mice were used in this experiment. Results from individual mice are shown. *, p < 0.05; **, p< 0.01 by one-way ANOVA.
Figure S4: Effect of E2 on CD40 and CD86 expression on cDCs and pDCs. Bone marrow cells from WT, ERα−/− or ERαAF10 were grown for 9 days with Flt3L in SFM supplemented or not with 10−9M E2. (A,B) The expression of CD40 (A) and CD86 (B) on cDC and pDC differentiated in absence (filled histogram) or in presence of E2 (black line). (C,D) Bar graphs show the geometric mean fluorescence for CD40 (C) and CD86 (D) on cDC and pDC. Results are presented as mean ± SD of triplicates, and are representative of three independent experiments. (*, p < 0.05).