The Journal of Immunology

RESEARCH ARTICLE | MARCH 15 2008

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J Immunol (2008) 180 (6): 3661–3669.
https://doi.org/10.4049/jimmunol.180.6.3661

Estrogen Receptor α, but Not β, Is Required for Optimal Dendritic Cell Differentiation and of CD40-Induced Cytokine Production[1]

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Dendritic cells (DC) are critical actors in the initiation of primary immune responses and regulation of self-tolerance. The steroid sex hormone 17β-estradiol (E_2) has been shown to promote the differentiation of DCs from bone marrow (BM) precursors in vitro. However, the estrogen receptor (ER) involved in this effect has not yet been characterized. Using recently generated ERα- or ERβ-deficient mice, we investigated the role of ER isotypes in DC differentiation and acquisition of effector functions. We report that estrogen-dependent activation of ERα, but not ERβ, is required for normal DC development from BM precursors cultured with GM-CSF. We show that reduced numbers of DCs were generated in the absence of ERα activation and provide evidence for a cell-autonomous function of ERα signaling in DC differentiation. ERα-deficient DCs were phenotypically and functionally distinct from wild-type DCs generated in the presence of estrogens. In response to microbial components, ERα-deficient DCs failed to up-regulate MHC class II and CD86 molecules, which could account for their reduced capacity to prime naive CD4$^+$ T lymphocytes. Although they retained the ability to express CD40 and to produce proinflammatory cytokines (e.g., IL-12, IL-6) upon TLR engagement, ERα-deficient DCs were defective in their ability to secrete such cytokines in response to CD40–CD40L interactions. Taken together, these results provide the first genetic evidence that ERα is the main receptor regulating estrogen-dependent DC differentiation in vitro and acquisition of their effector functions. *The Journal of Immunology,* **2008, 180: 3661–3669.**

Dendritic cells (DC)[4] are the major class of APCs. They play a central role in the initiation and coordination of the innate and adaptive immune responses by integrating signals from pathogens, cytokines, and T cells. DC activation can be induced by a variety of signals, such as microbial or viral products, which are directly recognized by members of the TLR family (1). Upon activation, DCs mature into potent APCs expressing high levels of MHC molecules and costimulatory molecules (CD80/CD86, CD40) and secrete immunomodulatory cytokines, such as IL-12, IL-6, and IL-10, that control the expansion and differentiation of naive T cells into effectors (2–4). Although IL-12 synthesis by DCs can be initiated by microbial signals, it requires reciprocal signaling from T cells for optimal production (5, 6). This cellular dialogue is mainly dependent on the interactions between CD40 expressed by DCs and its ligand CD154 (CD40L), which is expressed by CD4$^+$ T cells following TCR stimulation (7).

DCs represent an extremely plastic and versatile cell type, which plays a crucial role not only in the initiation and control of immunity and tolerance, but can also contribute to the induction of pathological situations such as autoimmune diseases (8). Although sex-based differences in the susceptibility to autoimmune diseases are well known, the underlying mechanisms are not understood (9). It has been shown that sex hormones, particularly estrogens, may contribute to the pathogenesis of some autoimmune diseases (9). The identification of estrogen receptors (ER) on immune cells suggested that sex steroid hormones, such as estrogens, may act directly on the immune system, modulating APC functions, lymphocyte activation, and/or cytokine-gene expression. Estrogen receptors α (ERα) and β (ERβ) belong to the nuclear receptor family of transcription factors. They are encoded by two different genes, *Esr1* and *Esr2*, and account for most of the known effects of estrogens (10). Human and mouse DCs express transcripts for both ER isotypes (11, 12) and could therefore represent a critical target for estrogens in vivo. Indeed, it has been shown that differentiation of DCs from murine bone marrow (BM) cells in the presence of GM-CSF was dramatically dependent on the presence of estrogens normally found in conventional culture medium (12). However,

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Received for publication November 27, 2007. Accepted for publication January 3, 2008.

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[1] This work was supported by grants from Agence Nationale de la Recherche (ANR-PHYSIO-06-010), Ligue Régionale contre le Cancer Région Midi-Pyrénées, Association Française contre les Myopathies, and Association pour la Recherche sur la Sclérose en Plaques.

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[4] Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; BMDC, BM-derived dendritic cell; CM, conventional medium containing phenol red and regular FCS; E_2, 17β-estradiol; ER, estrogen receptor; MHC-II, MHC class II; ODN, oligodeoxynucleotide; SFM, steroid-free medium; WT, wild type.

direct evidence for a role of ERα and/or ERβ signaling in this effect was still lacking.

In this study, we have attempted to elucidate the respective role of ERα and ERβ on GM-CSF-induced DC development and acquisition of effector functions, using recently generated ER-deficient mice (13). We confirmed the requirement for estrogens to generate optimal numbers of fully functional DCs in vitro, and we demonstrated that the effect of 17β-estradiol (E_2) on DC differentiation was dependent on ERα but not ERβ activation. The quantitative defect in DC development observed in the absence of ERα signaling was also associated with phenotypic and functional differences, as assessed by the expression of maturation markers, the ability to stimulate T cell proliferation and to secrete proinflammatory cytokines in response to TLR- or CD40-dependent stimulations. Taken together, these results show that E_2-dependent activation of ERα, but not ERβ, regulates critical steps involved in the development and acquisition of effector functions of DCs.

Materials and Methods

Mice

Female C57BL/6 (B6) (H-2^b, CD45.2) mice were purchased from Centre d'Elevage R. Janvier. ERα-deficient B6 mice (CD45.2), which have a deletion in the exon 2 of the ERα gene (ER$\alpha^{-/-}$), ERβ-deficient B6 mice (ER$\beta^{-/-}$), and littermate controls on B6 background have been previously described (13). Females were used in most experiments with ER-mutant mice, but identical results were obtained with males. CD45.1 B6.SJL congenic mice were initially obtained from The Jackson Laboratory. B10.D2 ER$\alpha^{-/-}$ (H-2^d) mice were generated in our own animal facilities by crossing ER$\alpha^{+/-}$ B6 mice with B10.D2 mice obtained from Harlan Sprague Dawley. After three backcrosses on B10.D2 background, ER$\alpha^{+/-}$ H-$2^{d/d}$ homozygotes were selected to generate B10.D2 ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ female mice. DO11.10 transgenic mice carrying a Vα2/Vβ8 TCR specific for OVA323–339/I-A^d complexes (14) on BALB/c (H-2^d) background were initially provided by Dr. L. Adorini (BioXell). Mice were bred and maintained in our specific pathogen-free animal facility. Protocols were approved by our institutional review board for animal experimentation.

DC generation from murine BM

BM-derived dendritic cells (BMDC) were generated as previously described (15). Briefly, BM cells were flushed out from femurs and tibias. After lysis of RBCs in ammonium chloride potassium, BM cells were cultured in conventional medium or steroid-free medium containing 20 ng/ml murine GM-CSF (PeproTech) at 2×10^5 cells/ml in bacteriological petri dishes (Greiner Bio-One). On day 3, an equal volume of fresh medium with 20 ng/ml GM-CSF was added to the culture, and on day 6, half of the medium was removed and replaced by fresh medium containing 10 ng/ml GM-CSF. Conventional medium (CM) was RPMI 1640 (Eurobio) supplemented with 10% heat-inactivated FCS (ATGC Biotechnologie), 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine, 50 μM 2-ME, and 50 μg/ml gentamicin (Sigma-Aldrich). Culture medium used for experiments in estrogen controlled conditions (referred to as steroid-free medium (SFM)) contained phenol red-free RPMI 1640 (Eurobio) with 10% dextran charcoal-treated FCS (HyClone) supplemented with 1 mM sodium pyruvate, 1% nonessential amino acids, 2 mM L-glutamine, 50 μM 2-ME, and 50 μg/ml gentamicin (Sigma-Aldrich). Cell treatments with E_2 (Sigma-Aldrich), with the ER antagonist $ICI_{182,780}$ (Tocris Bioscience), or with DMSO vehicle were performed at days 0, 3, and 6 of the cultures. Total cells in the culture were recovered at day 8 or day 9 and counted. DC yield was calculated by multiplying total cell number by the percentage of CD11c$^+$Gr-1$^-$ DCs in the culture, which was determined by flow cytometry as described below.

For mixed BM cultures, BM cells from CD45.1 mice (10^5 cells/ml) were mixed with equal amounts of CD45.2 ER$\alpha^{+/+}$ or CD45.2 ER$\alpha^{-/-}$ BM cells (10^5 cells/ml) and cultured with GM-CSF as described previously. Expressions of CD45.1 and CD45.2 alloantigens and of CD11c and Gr-1 markers were assessed by flow cytometry to calculate DC yields from each CD45 allotype.

DC purification and stimulations

DCs were purified from GM-CSF cultures by positive CD11c selection by preincubation with CD11c-specific microbeads and subsequent immunomagnetic sorting using MiniMACS columns (Miltenyi Biotec). Purity after enrichment was routinely between 80 and 95% CD11c$^+$ cells as assessed by flow cytometry. For stimulations with TLR agonists, purified DCs were stimulated with LPS (*Escherichia coli* 0111:B4 LPS Ultrapure; InvivoGen), poly(I:C) (Sigma-Aldrich), CpG-containing phosphorothioate oligodeoxynucleotide (ODN) 1668 (Sigma-Aldrich), or GpC-ODN control (Sigma-Aldrich). For CD40-dependent stimulation, purified DCs were cocultured with control mock-transfected or CD40L (CD154)-expressing National Institutes of Health 3T3 fibroblasts, which were a gift from Dr. P. Hwu (National Cancer Institute, Bethesda, MD) and were provided by Dr. C. Reis e Sousa (Cancer Research U.K., London).

Analysis of surface markers and cytokine production

Before staining, cells ($5–10 \times 10^5$) 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-, biotin-, or APC-conjugated mAbs diluted at the optimal concentration in FACS buffer (PBS 1% FCS, 5 mM EDTA, 1‰ NaN_3). When biotinylated mAbs were used, cells were washed twice in FACS buffer before incubation with APC-conjugated streptavidin (eBioscience). The following mAbs for cell surface staining were purchased from BD Biosciences: anti-CD11c (HL3), anti-CD11b (M1/70), anti-Ly-6C (AL-21), anti-CD86 (GL1); or from eBioscience: anti-CD11c (N418), anti-MHC class II (M5/114.15.2), anti-CD40 (HM40-3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-TLR4/MD2 (MTS510), or anti-CD4 (GK1.5). Flow cytometry analyses were performed on a FACSCalibur flow cytometer (BD Biosciences).

For phenotypic analysis of DC maturation and intracellular cytokine production, purified DCs were incubated in CM supplemented with 10 ng/ml GM-CSF and stimulated for 18 h with 2 μg/ml LPS. DCs were recovered by incubation for 15 min on ice with PBS containing 1% FCS and 2 mM EDTA. For detection of intracellular cytokine production, DCs stimulated as indicated above were incubated with 10 μg/ml brefeldin A (Sigma-Aldrich) for the last 4 h of culture. After surface staining with FITC-anti-MHC-II and APC-anti-CD11c and fixation in PBS 1% paraformaldehyde, cells were permeabilized with 0.5% saponin, and intracellular cytokine staining was performed with PE-anti-IL-6 (MP5–20F3), FITC-anti-TNF-α (MP6-XT2), PE-anti-IL-12p40/p70 (C15.6), or PE-rat IgG1 isotype control, all from BD Biosciences.

For cytokine production, DCs were cultured in 96-well plates (3×10^4 cells/well) and stimulated with 2 μg/ml LPS, 10 μg/ml poly(I:C), 1 μg/ml CpG-ODN, or 1 μg/ml GpC-ODN control. For CD40-dependent stimulation, DCs (6×10^4 cells/well) were cocultured with CD40L-transfected National Institutes of Health 3T3 fibroblasts (2.5×10^4 cells/well) in 96-well plates in the absence or presence of anti-CD154 mAb (BD Biosciences). Mock-transfected National Institutes of Health 3T3 fibroblasts were used as control. To assess IL-12p70 production, 5 ng/ml IFN-γ (PeproTech) was added to the stimulations. Production of IL-6, TNF-α, and IL-12p40 were measured in 24-h culture supernatants, and IL-12p70 was measured in 48-h culture supernatants. Cytokines were quantified by two-site sandwich ELISA (all mAbs were purchased from BD Biosciences).

Assessment of Ag-specific CD4$^+$ T cell activation

The ability of DCs to activate Ag-specific T cells was monitored by measuring CFSE dilution and thymidine incorporation of OVA-specific CD4$^+$ T cells from DO11.10 TCR transgenic mice. CD4$^+$ T cells were enriched by negative selection using CD4$^+$ T cell isolation kit (Dynal Biotech) and labeled with 5 μM CFSE as described elsewhere (16). CFSE-labeled DO11.10 CD4$^+$ T cells were incubated at 1×10^5 cells per well in 96-well plates (Costar) with a constant number of CD11c-sorted ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ B10.D2 DCs (3×10^4 cells) per well and titrated concentrations of endotoxin-free OVA protein (Sigma-Aldrich) or $OVA_{323–339}$ peptide (NeoMPS). Cells were cultured in CM at 37°C in a humidified atmosphere containing 5% CO_2. After 72 h culture, cell division was assessed by flow cytometry. DO11.10 TCR transgenic CD4$^+$ T cells labeled with CFSE were stained with biotinylated anti-DO11.10 clonotype KJ1.26 and PE-conjugated anti-CD4. To assess CD4$^+$ T cell proliferation, cultures were set up as above and pulsed with 1 μCi [^{3}H]TdR (40 Ci/nmol, the Radiochemical Centre, Amersham, U.K.) at 48 h. Incorporation of [^{3}H]TdR was measured 12 h later by using a MicroBeta TriLux luminescence counter (PerkinElmer).

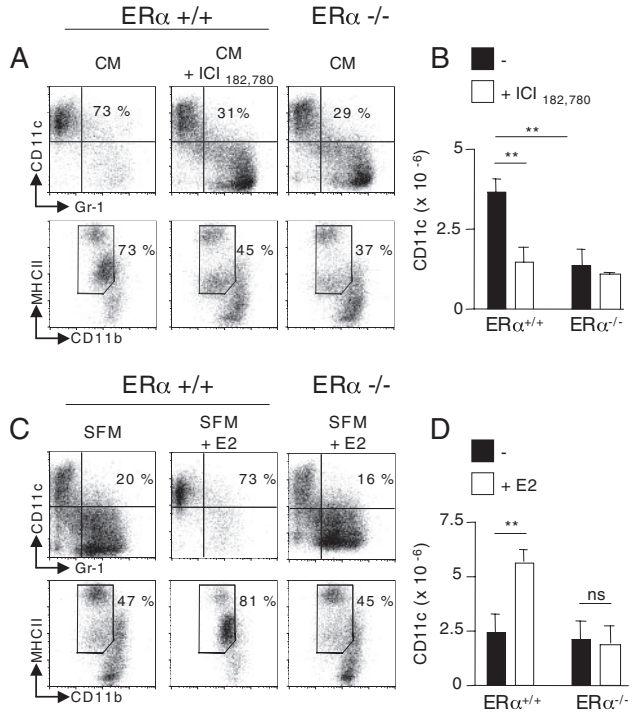

FIGURE 1. ERα activation is critical for BMDC generation. ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ BM cells were grown for 9 days with GM-CSF in CM supplemented or not with 20 nM $ICI_{182,780}$ (*A* and *B*) or in SFM supplemented or not with 10 nM E_2 (*C* and *D*). The percentages of $CD11c^+Gr\text{-}1^-$ and $CD11b^{int}MHC\text{-}II^+$ cells of gated $CD11c^+$ cells were determined by flow cytometry and are indicated. Absolute number of DCs generated in CM (*B*) or in SFM (*D*) are reported as the means ± SEM of five to seven independent experiments. Differences between variables were evaluated by the Mann-Whitney *U* test (**, $p < 0.01$; ns, not significant).

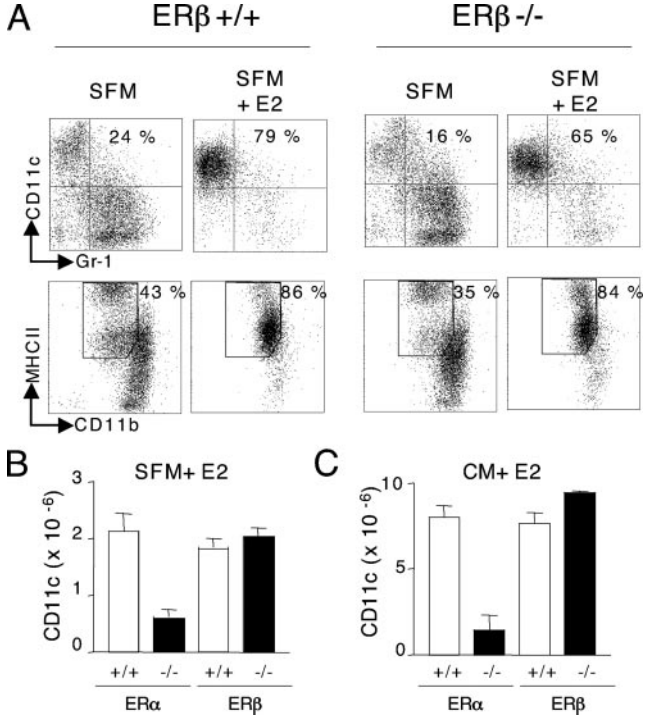

FIGURE 2. ERβ activation is dispensable for BMDC differentiation. *A*, ER$\beta^{-/-}$ or ER$\beta^{+/+}$ BM cells were grown for 9 days with GM-CSF in SFM supplemented or not with 10 nM E_2. The percentages of $CD11c^+Gr\text{-}1^-$ and $CD11b^{int}MHC\text{-}II^+$ cells of $CD11c^+$-gated cells were determined by flow cytometry and are indicated. Absolute numbers of DCs generated from ER$\alpha^{-/-}$ or ER$\beta^{-/-}$ BM cells in SFM (*B*) or in CM (*C*) supplemented with 10 nM E_2 are expressed as the means ± SEM of triplicate or quadruplicate cultures. Data are representative of three experiments.

Results

Generation of DC from BM progenitors is impaired in absence of E_2 and requires ERα- but not ERβ-dependent signaling

Culture of BM cells in the presence of GM-CSF leads to the differentiation of $CD11c^+$ myeloid DCs, expressing CD11b and high to intermediate levels of MHC class II (MHC-II) molecules (15, 17). Using this culture system, it has been previously shown that the absence of estrogens or the presence of ER antagonists resulted in an impaired development of $CD11c^+CD11b^{int}$ DCs that normally represent most cells generated in estrogen-supplemented medium (12). Instead, culture of BM cells in steroid hormone-deficient medium generated mainly CD11c-negative cells that express the myeloid differentiation marker Gr-1 and low to high levels of CD11b (12). In the present study, we used this culture system to determine the role of ER isotypes in this effect of E_2 on DC differentiation using recently generated ERα- or ERβ-deficient mice (13). BM cells from ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ littermate control mice were culture in CM in the presence or absence of the pure ER antagonist $ICI_{182,780}$. As shown in Fig. 1, the frequency as well as the absolute number of $CD11c^+Gr\text{-}1^-$ DCs that developed from ER$\alpha^{-/-}$ BM cultures was reduced up to 3- to 4-fold as compared with wild-type (WT) BM. This quantitative defect was associated with phenotypic changes between WT and ER$\alpha^{-/-}CD11c^+$ DCs as shown by the analysis of CD11b and MHC-II expression (Fig. 1, *A* and *C*). Whereas WT $CD11c^+$ DCs were mainly composed of $CD11b^{int}MHC\text{-}II^{int/high}$ cells, $CD11c^+$ cells from ER$\alpha^{-/-}$ BM cultures were enriched in cells expressing higher levels of CD11b and low to intermediate levels of MHC-II molecules ($MHC\text{-}II^{low/int}$). To control the implication of estrogens present in standard culture medium, the pure ER antagonist $ICI_{182,780}$ (2×10^{-8} M) was added to the cultures at days 0, 3, and 6 (Fig. 1, *A* and *B*). As expected, blocking the endogenous stimulation of ER reduced the development of DCs from WT BM cells (Fig. 1, *A* and *B*). Furthermore, DCs generated under such conditions exhibited a CD11b/MHC-II phenotype indistinguishable from ERα-deficient $CD11c^+$ cells (Fig. 1*A*). In agreement with previous works (12), similar results were obtained when DCs were generated in steroid hormone-deficient medium (Fig. 1, *C* and *D*). Addition of E_2 (10 nM) in cultures of WT but not ER$\alpha^{-/-}$ BM cells effectively restored the capacity of the BM progenitors to generate normal numbers of DCs with the expected phenotype (Fig. 1, *C* and *D*).

Although our data demonstrate the obligatory role of ERα in promoting DC development, it has been previously suggested that ERβ could also be implicated in DC differentiation from BM precursors (12). To address this point, BMDCs were generated from ER$\beta^{-/-}$ or ER$\beta^{+/+}$ progenitors in steroid-free medium supplemented or not with E_2 (10 nM). Absence of E_2 led to an impaired development of $CD11c^+$ DCs in both ER$\beta^{+/+}$ and ER$\beta^{-/-}$ BM cell cultures that exhibited a CD11b/MHC-II phenotype similar to ER$\alpha^{-/-}$ DCs (Fig. 2*A*). Addition of E_2 to the steroid-free cultures allowed ER$\beta^{-/-}$ BM progenitors to differentiate into DCs as efficiently as ER$\beta^{+/+}$ or ER$\alpha^{+/+}$ control cells (Fig. 2, *A* and *B*). Again, E_2 supplementation of ER$\alpha^{-/-}$ BM cultures could not restore normal numbers of $CD11c^+$ DCs, in agreement with data in Fig. 1. Similar results were obtained when BMDCs were generated in CM containing regular FCS and thereby E_2 (Fig. 2*C*). Taken

together, these data demonstrate that estrogens are required to support efficient DC development from BM precursors in vitro through ERα but not ERβ.

Deficiency of ER$\alpha^{-/-}$ BM cells to develop into DCs is a cell-autonomous feature

As ERα signaling has been shown to regulate cytokine production in myeloid cells in vitro (18, 19), it was important to distinguish whether the impaired DC development was caused by a cell-intrinsic defect of ERα signaling within the DC lineage or by an indirect effect due to autocrine or paracrine factors, which could regulate DC development. We examined the generation of CD11c$^+$ DCs from either ER$\alpha^{+/+}$ or ER$\alpha^{-/-}$ Ly-5.2 BM cells when cocultured with equal numbers of Ly-5.1 WT BM progenitors in CM supplemented or not with an excess of E_2 (Fig. 3). As shown in Fig. 3*A*, the proportion of CD11c$^+$Gr-1$^-$ DCs expressing the CD45.2 allotypic marker was reduced by >2-fold when ER$\alpha^{-/-}$ CD45.2 BM cells were cultured in competition with WT CD45.1 cells. This difference was even exacerbated in E_2-supplemented medium, indicating that a high dose of E_2 further promoted DC development in WT but not in ER$\alpha^{-/-}$ BM progenitors (Fig. 3, *B* and *E*). Additionally, analysis of the CD11b/MHC-II expression profile of ER$\alpha^{-/-}$ DCs (CD45.2) generated in the presence of WT CD45.1 progenitors (Fig. 3*C*) exhibited a similar phenotype as ER$\alpha^{-/-}$ DCs generated alone (see Fig. 1). To better define the DC subsets generated under these various conditions, we also assessed the relative expression of CD11b and Ly-6C among CD11c$^+$ cells. Indeed, E_2 has been shown to preferentially promote the differentiation of CD11c$^+$CD11b^{int} lacking Ly-6C expression, whereas the proportion of CD11b^{high}Ly-6C$^+$ cells among CD11c$^+$ cells was increased in the absence of E_2 (20). We could identify CD11b^{high}Ly-6C$^+$ and CD11b^{int}Ly-6C$^-$ subsets in both WT and ER$\alpha^{-/-}$ DC cultures (Fig. 3, *D* and *E*). The frequency of CD11b^{high}Ly-6C$^+$ cells was increased in DCs developing from ER$\alpha^{-/-}$ progenitors. Similar results were obtained when DCs were generated from WT BM in the absence of E_2 (not shown). Ly-6C$^-$CD11c$^+$ cells expressing an intermediate and homogenous level of CD11b (CD11b^{int}Ly-6C$^-$) were the most frequent subset in the progeny of ER$\alpha^{+/+}$ BM cells. By determining the number of DCs generated in each combination, we observed that the absolute number of CD11b^{int}Ly-6C$^+$ among CD45.1/CD45.2 was neither affected by the presence of a functional ERα gene in BM precursors nor by providing excess E_2 during DC differentiation (Fig. 3*E*). By contrast, the generation of CD11b^{int}Ly-6C$^-$ DCs, which represented most CD11c$^+$ cells from WT BM cultures, was strongly dependent on ERα signaling. Indeed, when cocultured with ER$\alpha^{-/-}$ cells, ER$\alpha^{+/+}$ (CD45.1$^+$) cells represented 75–87% of total CD11b^{int}Ly-6C$^-$CD11c$^+$ in the absence or presence of exogenously added E_2, respectively. Thus, the generation of CD11b^{int}Ly-6C$^-$ DCs from ER$\alpha^{-/-}$ BM precursors could not be rescued by WT hemopoietic progenitors. Reciprocally, the development of WT CD45.1 DCs was not affected by the presence of ERα-deficient BM cells. Taken together, these results further underscore a requirement for ERα activation in DC development and provide evidence for a cell-autonomous function for ERα signaling in DC generation.

ERα-deficient DCs show phenotypic and functional abnormalities

DC development is decreased in the absence of ERα signaling, but it is not abolished. The DCs that develop in these conditions are enriched in cells expressing high levels of CD11b, Ly-6C, and lower levels of MHC-II that may represent immature myeloid DCs. ER$\alpha^{-/-}$ DCs were enriched in cells expressing low to undetectable levels of MHC-II molecules and displaying high CD11b staining (see Figs. 1–3; Fig. 4*A*). Although expression of costimulatory molecules was similar between most untreated immature ER$\alpha^{-/-}$ and control DCs, the frequency of cells expressing high levels of MHC-II and CD86 was higher in WT DCs than in ER$\alpha^{-/-}$ DCs (Fig. 4*A*). DCs were stimulated through TLR4 (LPS) or TLR9 (CpG-DNA) for 24 h, followed by flow cytometric assessment of surface expression of MHC-II, CD86, and CD40 costimulatory molecules. As expected, a strong up-regulation of MHC-II and costimulatory molecules CD86 or CD40 was observed in WT DCs after stimulation with LPS or CpG-DNA. By contrast, despite an increased expression of CD40 molecules to levels similar to WT DCs, ~30–50% of ER$\alpha^{-/-}$ DCs failed to

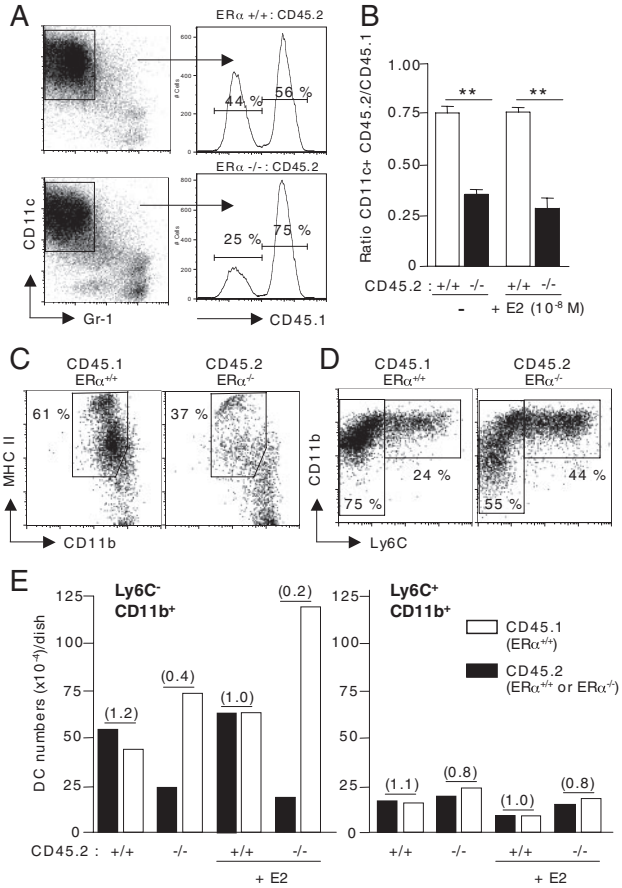

FIGURE 3. Intrinsic expression of ERα is required to promote BMDC development. *A* and *B*, CD45.2 BM cells from ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ mice (3 mice/group) were cultured in competition with CD45.1 BM cells at a 1:1 ratio in CM alone or supplemented with 10 nM E_2. *A*, Percentages of CD45.1$^+$ and CD45.1$^-$ cells (CD45.2$^+$, not shown) gated on CD11c$^+$Gr-1$^-$ DCs are indicated. *B*, Ratios between the frequency of CD45.1 and CD45.2 DCs (defined above as CD45.1$^-$ DCs) in each combination are indicated. Data are expressed as means ± SEM of 3 mice/group. Data were analyzed for statistical significance with a two-tailed Student's *t* test (**, $p < 0.01$). *C* and *D*, The expression of phenotypic (CD11c, Gr-1, CD11b, MHC-II, Ly-6C) and allotypic CD45.1 markers was analyzed in competition experiment performed as in *A*. The percentages of CD11b^{int}MHCII$^+$ (*C*) and CD11b$^+$Ly-6C$^-$ or Ly-6C$^+$ DCs (*D*) among WT CD45.1 and ER$\alpha^{-/-}$ CD45.2 DCs are shown. *E*, The absolute numbers of CD11b$^+$Ly-6C$^-$ and CD11b$^+$Ly-6C$^+$ DCs generated from WT CD45.1 BM cells cultured in competition with ER$\alpha^{-/-}$ or control ER$\alpha^{+/+}$ CD45.2 BM cells are indicated. Cultures were performed in CM alone or supplemented with an excess of E_2 as indicated. Numbers in parentheses indicate the ratio between CD45.2 and CD45.1 DCs. Data are representative of three independent experiments.

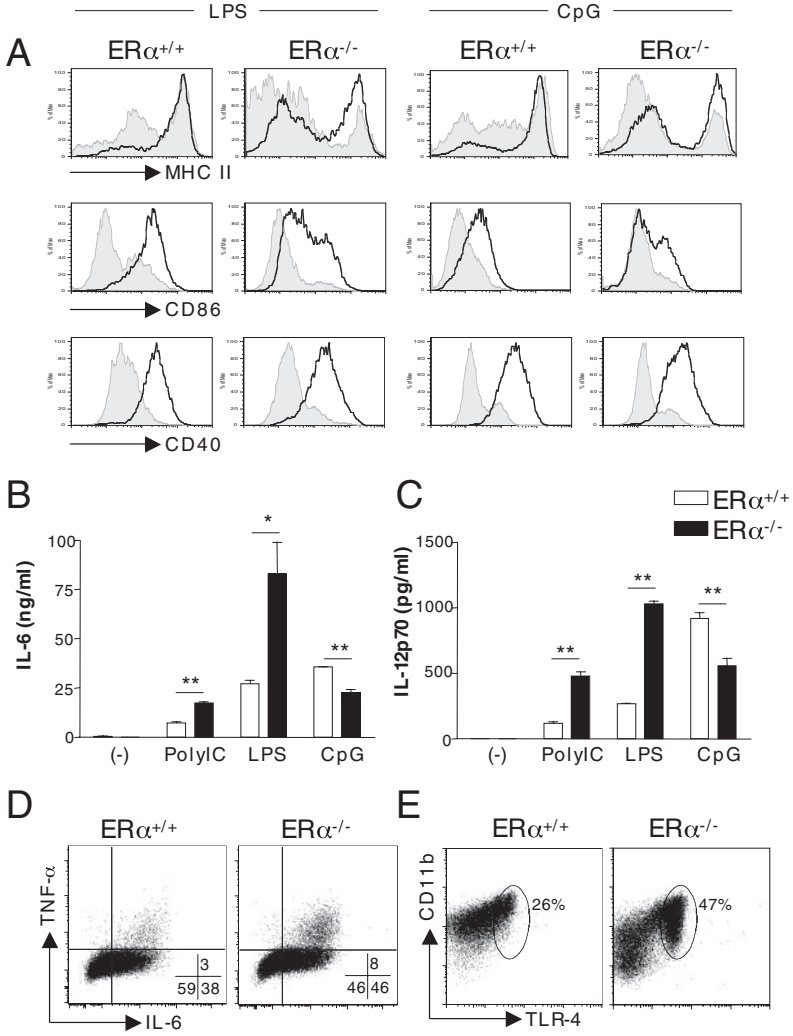

FIGURE 4. ER$\alpha^{-/-}$ BMDCs are phenotypically and functionally distinct from WT BMDCs. ER$\alpha^{+/+}$ or ER$\alpha^{-/-}$ BMDCs were generated in CM as in Fig. 1 and were purified by CD11c-positive selection. *A*, DCs were left untreated (filled histogram) or stimulated for 18 h with TLR ligands as indicated (open histogram). Expression of MHC-II, CD86, and CD40 were analyzed by flow cytometry. *B* and *C*, ER$\alpha^{+/+}$ or ER$\alpha^{-/-}$ DCs were stimulated with poly(I:C), LPS, or CpG-DNA in the absence (*B*) or presence of IFN-γ (*C*). IL-6 and IL-12p70 were measured by ELISA in 24 (*B*) or 48 h (*C*) culture supernatants, respectively. Results are expressed as the means $\pm$ SEM of 3 mice/group. Data were analyzed for statistical significance with a two-tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). *D*, DCs were stimulated with LPS (2 μg/ml) for 18 h and were assessed for IL-6 and TNF-α production by intracellular staining. Dot plots show IL-6 vs TNF-α staining of gated CD11c$^+$ cells. *E*, Dot plots show CD11b vs TLR4 expression of resting CD11c$^+$ ER$\alpha^{+/+}$ or ER$\alpha^{-/-}$ DCs. Data are representative of three to four independent experiments.

up-regulate MHC-II or CD86 molecules upon stimulation through TLR4 or TLR9 (Fig. 4*A*). Thus, as for MHC-II molecules, up-regulation of CD86 was significantly impaired in some ER$\alpha^{-/-}$ DCs in response to LPS or CpG. By contrast, no major defect in CD40 expression was observed after LPS- or CpG-induced maturation of ER$\alpha^{-/-}$ DCs.

We also measured IL-6 and IL-12 production by DCs stimulated with poly(I:C) (TLR3), LPS, and CpG-DNA in the presence or absence of IFN-γ. As shown in Fig. 4*B*, ER$\alpha^{-/-}$ DCs stimulated with LPS or poly(I:C) secreted more IL-6 and TNF-α (not shown) than did WT DCs, whereas cytokine production in response to CpG was slightly reduced in ER$\alpha^{-/-}$ DCs (Fig. 4*B*). Likewise, in the presence of IFN-γ, IL-12p70 secretion was again superior in ER$\alpha^{-/-}$ DC cultures stimulated with LPS or poly(I:C) as compared with WT DCs. Thus, despite some defects in their maturation processes, ER$\alpha^{-/-}$ DCs exhibited an enhanced capacity to produce various proinflammatory cytokines in response to microbial components that trigger DCs through TLR4 or TLR3. This observation was confirmed by single-cell analysis of IL-6 and TNF-α production by intracellular staining after LPS stimulation. DCs producing either IL-6, TNF-α, or both were more frequent in CD11c$^+$ER$\alpha^{-/-}$ DCs stimulated by LPS (Fig. 4*D*). This enhanced LPS responsiveness of ER$\alpha^{-/-}$ DCs was correlated with an increased frequency of cells expressing high levels of TLR4 and CD11b molecules (Fig. 4*E*).

T cell stimulatory capacity of ER$\alpha^{-/-}$ DCs is impaired

Because the principal function of DCs is to activate T lymphocytes, we next evaluated the ability of ER$\alpha^{-/-}$ DCs to prime OVA-specific naive CD4$^+$ T cells from DO11.10 Tg mice. For this purpose, the ERα mutation was backcrossed to B10.D2 mice to generate ER$\alpha^{-/-}$ mice of the H-2^d haplotype. The defect in BMDC development was identical between B10.D2 and C57BL/6 ERα-deficient mice (data not shown). DCs were generated from B10.D2 ER$\alpha^{-/-}$ or ER$\alpha^{+/+}$ mice, and CD11c$^+$-purified DCs were then used to stimulate transgenic DO11.10 CD4$^+$ T cells that express a TCR specific for the I-A^d/OVA$_{323–339}$ peptide complex. The proliferative capacity of DO11.10 CD4$^+$ T cells was significantly impaired when ER$\alpha^{-/-}$ DCs were used as APCs in response to both OVA$_{323–339}$ peptide (Fig. 5, *A* and *B*) or OVA protein (Fig. 5, *C* and *D*). We next determined whether the defective capacity of ERα-deficient DCs to prime OVA-specific CD4$^+$ T cell proliferation was due to lack of E_2-mediated signaling during DC development. B10.D2 DCs were generated in steroid-free medium supplemented or not with various doses of E_2. Purified DCs were then tested for their capacity to activate DO11.10 T cells in the presence of OVA$_{323–339}$ peptide. As shown in Fig. 5*E*, WT DCs generated in the absence of E_2, like ERα-deficient DCs, exhibited a reduced capacity to induce the proliferation of DO11.10 CD4$^+$ T cells. This functional defect was reversed by adding

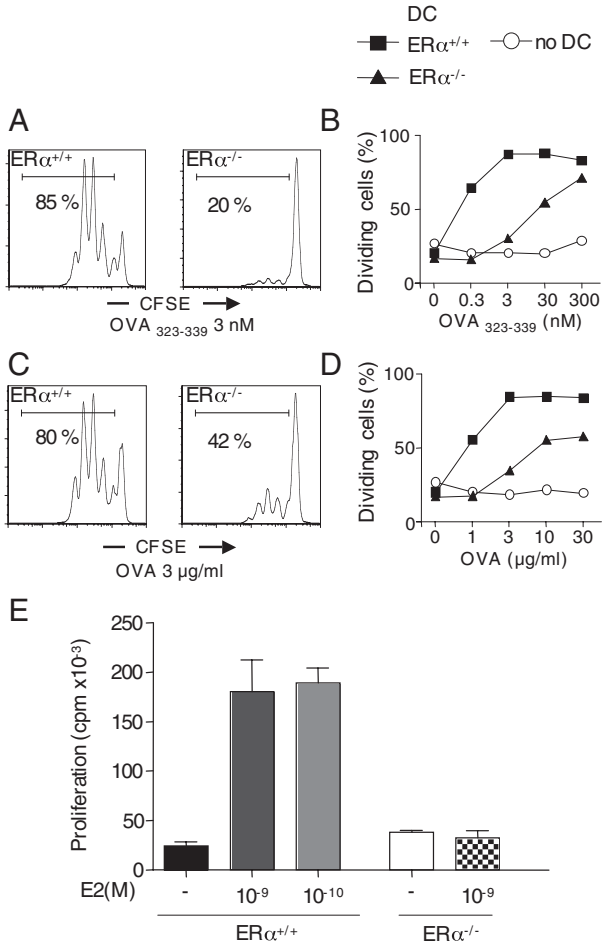

FIGURE 5. ERα activation during DC development enhances the $CD4^+$ T cell-priming capacity of DCs. Purified $CD11c^+$ $ER\alpha^{+/+}$ or $ER\alpha^{-/-}$ BMDCs generated in CM as in Fig. 1 were loaded with $OVA_{323–339}$ peptide (*A*, *B*) or with OVA protein (*C*, *D*) to stimulate naive transgenic DO11.10 $CD4^+$ T cells. Histograms show CFSE intensity of $KJ1.26^+CD4^+$ T cells after 72 h stimulation (*A*, *C*). Results are expressed as percentage of dividing cells among $KJ1.26^+CD4^+$ T cells (*B*, *D*) and are representative of three independent experiments. *E*, BMDC cultures were conducted in SFM supplemented or not with the indicated doses of E_2. $ER\alpha^{+/+}$ or $ER\alpha^{-/-}$ $CD11c^+$ DCs were purified and were loaded with 0.1 μM OVA peptide to stimulate DO11.10 $CD4^+$ T cells. $CD4^+$ T cell proliferation was assessed by [^{3}H]thymidine incorporation during the last 12 h of culture. Results are presented as the means ± SEM of quadruplicate cultures and are representative of three to four independent experiments.

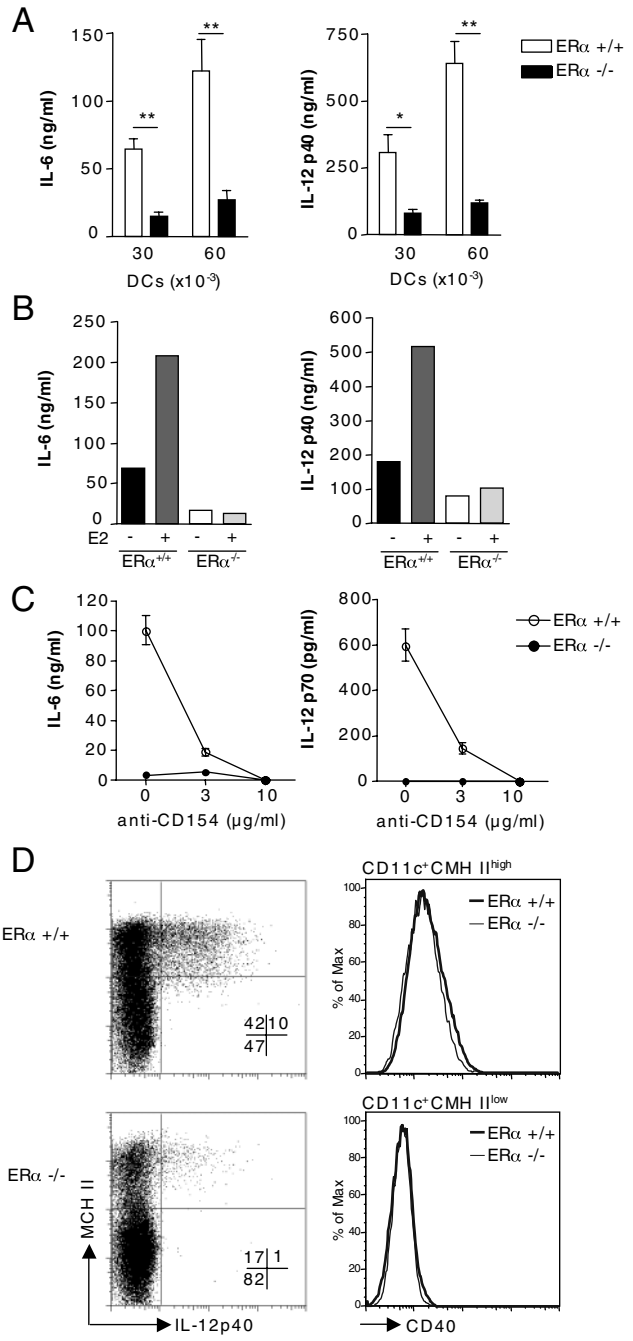

FIGURE 6. $ER\alpha^{-/-}$ BMDCs exhibit impaired production of proinflammatory cytokines in response to CD40 triggering. *A*, Purified $CD11c^+$ BMDCs from $ER\alpha^{+/+}$ or $ER\alpha^{-/-}$ mice were cultured on a monolayer of CD40L-expressing fibroblasts. IL-6 and IL-12p40 were measured by ELISA in 24-h culture supernatants. Data are expressed as the means ± SEM of 4 mice/group and were analyzed for statistical significance with a two-tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). *B*, E_2 (10^{-8} M) was added or not during BMDC differentiation in CM, and the production of IL-6 and IL-12p40 by CD40L-stimulated DCs was tested as above. *C*, DCs (30×10^3 DCs per well) obtained as in *B* in CM with E_2 from $ER\alpha^{+/+}$ or ER $\alpha^{-/-}$ BM cells were cultured on a monolayer of CD40L-expressing fibroblasts with IFN-γ (5 ng/ml) to measure IL-6 and IL-12p70 production in 24-h culture supernatants. Anti-CD154 mAb was added to the cultures at the indicated doses. *D*, DCs obtained as in *C* were stimulated during 18 h with CD40L-expressing cells. Cells were stained with mAb specific for CD11c, CD40, and MHC-II and fixed for intracellular analysis of IL-12p40 production by flow cytometry. *Left*, Dot plots show IL-12 p40 vs MHC-II of gated $CD11c^+$ DCs. *Right*, Expression of CD40 on MHC-II^{high} or MHC-II^{low} $CD11c^+$ DCs. Results are representative of two to three independent experiments.

exogenous E_2 to WT but not to $ER\alpha^{-/-}$ DCs. Indeed, when DCs were generated in the presence of doses ranging from 0.1 to 10 nM, they were able to efficiently activate naive $CD4^+$ T cells (Fig. 5*E* and data not shown). This was confirmed by analyzing CFSE dilution in DO11.10 T cells (data not shown). E_2 at 0.01 nM or below could not support efficient DC development, and DCs generated in this condition had a phenotype similar to $ER\alpha^{-/-}$ DCs (data not shown).

DCs that develop in the absence of ER signaling have reduced cytokine response to CD40 triggering

Because DC effector functions are markedly dependent on T cell-derived signals (5, 6), we assessed the effect of CD40 ligation on the cytokine response of WT or $ER\alpha^{-/-}$ DCs. We showed that CD40 expression was similar between immature WT and $ER\alpha^{-/-}$ DCs and was strongly up-regulated in both DC populations upon

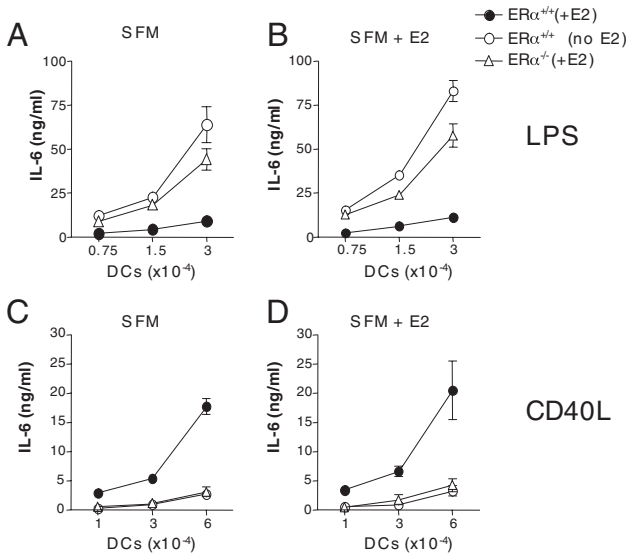

FIGURE 7. ERα activation during DC development controls the acquisition of specific effector functions. ER$\alpha^{+/+}$ BM cells were cultured for 9 days in SFM supplemented with 10 nM E_2 (●) or with vehicle (DMSO, ○). ER$\alpha^{-/-}$ BMDCs were generated in SFM with 10 nM E_2 (△). Purified CD11c$^+$ DCs were stimulated with LPS (*A* and *B*) or CD40L-transfected cells (*C* and *D*) in SFM (*A* and *C*) or SFM supplemented with 10 nM E_2 (*B* and *D*). IL-6 production was measured in 24-h culture supernatants by ELISA. Results are presented as the means ± SEM of triplicate cultures and are representative of three experiments.

stimulation with LPS or CpG (Fig. 4). We next evaluated the capacity of DCs to respond to CD40-dependent signaling. Culturing WT DCs on a monolayer of CD40L-expressing fibroblasts, but not control cells (not shown), induced high levels of IL-6 and IL-12p40 (Fig. 6*A*). In contrast, cytokine production was strongly reduced in ER$\alpha^{-/-}$ DCs upon CD40 triggering (Fig. 6*A*). Addition of an excess of E_2 during DC development resulted in an enhanced production of IL-6 and IL-12p40 in WT but not in ER$\alpha^{-/-}$ DCs (Fig. 6*B*). Similar results were obtained upon CD40L stimulation in the presence of IFN-γ (Fig. 6*C*). In addition to IL-6, high levels of IL-12p70 were induced in WT DCs but not in ER$\alpha^{-/-}$ DCs. Cytokine production by DCs was blocked in the presence of anti-CD154 Ab (Fig. 6*C*).

We then determined the frequency of IL-12p40-producing cells by intracellular staining of DCs stimulated with CD40L-transfected fibroblasts for 18 h. Compared with baseline staining with an isotype control mAb, 10–25% of WT DCs could be stained for IL-12p40 (Fig. 6*D* and data not shown). In contrast, the frequency of IL-12p40-producing cells was reduced by 3- to 10-fold in ER$\alpha^{-/-}$ DCs (Fig. 6*D* and data not shown). Cytokine-producing cells were contained in DCs expressing high levels of MHC-II in both CD40L-stimulated WT and ER$\alpha^{-/-}$ DCs. DCs that had up-regulated MHC-II molecules (MHC-II^{high}) had also increased their expression of CD40 when compare with MHC-II^{low} DCs. Notably, no differences were seen between WT and ER$\alpha^{-/-}$ DCs, which indicates that defective CD40L-mediated activation of ER$\alpha^{-/-}$ DCs cannot be explained by a reduced expression of CD40 molecules.

Finally, we evaluated whether the functional differences we observed in ER$\alpha^{-/-}$ DCs were also found in DCs generated from WT progenitors in the absence of estrogens. Purified WT DCs generated in steroid-free medium supplemented or not with E_2 were activated for 24 h with LPS (Fig. 7, *A* and *B*) or CD40L-transfected cells (Fig. 7, *C* and *D*) in the absence (Fig. 7, *A* and *C*) or presence of E_2 (Fig. 7, *B* and *D*). As shown in Fig. 7*A*, IL-6 synthesis was strongly enhanced in LPS-stimulated ER$\alpha^{-/-}$ DCs but also in WT DCs generated in the absence of E_2 (E_2-deprived DCs) as compared with WT DCs generated in E_2-supplemented medium. When stimulated through CD40, again ER$\alpha^{-/-}$ DCs and E_2-deprived WT DCs had an identical phenotype and produced significantly less IL-6 as compared with E_2-supplemented WT DCs (Fig. 7*C*). Similar cytokine profiles were observed when DC stimulations were performed in E_2-supplemented medium (Fig. 7, *B* and *D*). Thus, the presence of the hormone at the time of TLR- or CD40-mediated stimulation had little if any effect on cytokine production by DCs. These results are consistent with an E_2 action, through ERα, on precursor cells during DC development rather than on already differentiated cells.

Discussion

In the present study, we confirm that estrogens are critical to support normal DC development from BM precursors (12), and we unambiguously establish that ERα, but not ERβ, is required to mediate this effect. Indeed, DCs generated from WT precursors grown in steroid-free conditions were indistinguishable from DCs derived from ERα-deficient precursors irrespective of the presence or absence of E_2. ERα-deficient DCs showed an impaired capacity to up-regulate MHC-II and CD86 molecules upon TLR stimulation and to activate naive CD4$^+$ T cells. Failure of ER$\alpha^{-/-}$ DCs to efficiently prime CD4$^+$ T cells was associated with a reduced ability to produce proinflammatory cytokines in response to CD40L. Thus, E_2-dependent activation of ERα, but not ERβ, regulates critical steps involved in DC development in vitro.

It has been previously shown by Kovats and coworkers that estrogens were required to promote DC differentiation from BM progenitors, but the respective roles of ERα and ERβ in this effect remained unresolved (12). Of note, Kovats's group previously used a first-generation model of ERα-targeting mice, consisting of the insertion of a Neo cassette into exon 1 (hereafter called ERα-Neo$^{-/-}$) (21). Although the development of DCs from ERα-Neo$^{-/-}$ mice was impaired, addition of excess of E_2 restored near normal numbers of CD11c$^+$CD11b^{int} cells in the cultures, suggesting a possible compensatory role of ERβ (12). The explanation of this apparent discrepancy resides most likely in the recently characterized phenotypic difference between these two mutant strains. Although the expression of the full-length 66 kDa isoform of ERα (p66) is abolished in ERα-Neo$^{-/-}$ mice, two others splice variants lacking the AF-1 transactivator domain have been identified (p55, p46) that still possess a residual estrogen-dependent transcriptional activity (22–24). In contrast, in the mouse model of complete inactivation of ERα used in the present study (13, 22), E_2, even in high amounts, failed to promote DC differentiation from BM progenitors, demonstrating that ERβ signaling could not compensate for the lack of ERα. Thus, our results show for the first time that ERα is the main receptor implicated in the E_2-dependent differentiation of BM progenitors into DCs in vitro. These data also suggest that the AF-1 transactivator domain of ERα might be dispensable for the E_2-mediated effect on DC development as it was previously shown for some vascular effects of E_2 (22).

Myeloid progenitors can be distinguished into several subsets according to CD34 and CD16/32 expression, among them a CD34$^+$CD16/32$^+$ common precursor for both macrophages and tissue resident DCs has been recently identified, based on the expression of CX3CR1 (25). We showed that inhibition of ERα activation in WT BM cells during DC differentiation led to a phenotype similar to that of ER$\alpha^{-/-}$ cells excluding specific myeloid precursor deficiency due to lack of estrogen signaling in ER$\alpha^{-/-}$

mice in vivo. Moreover, it was previously shown that E_2 had maximal effect at the beginning of the culture, consistent with E_2 action on precursor cells (12). Additionally, impaired DC development persisted when ERα-deficient progenitors were cocultured with WT cells, indicating a cell-intrinsic requirement for ERα activation. Likewise, the generation of WT DCs was not affected by the presence of ERα-deficient progenitors. Thus, default DC development from ER$\alpha^{-/-}$ progenitors was intrinsic to the cells and not due to autocrine or paracrine effects of cytokines present in the microenvironments. Activated ligand-bound ER classically leads to genomic effects. Transcriptional responses to estrogens were initially recognized to depend on specific interaction of activated ER with ERE sequences in the promoter of target genes, but interaction of ER with other transcription factor complexes, like AP-1 (26) or Sp-1 (27), are common modulating mechanisms of their transcriptional activity. Although the transcription factor families AP-1 and Sp-1 are ubiquitously expressed, they are known to regulate several myeloid-specific gene expressions (28, 29). Our current hypothesis is that E_2-dependent activation of ERα might regulate the activation state or expression level of transcription factors implicated in DC lineage commitment at early stages during differentiation of BM precursors (30). Interestingly, it has been recently shown that E_2 acts directly on highly purified myeloid progenitors, including the CX3CR1+ common macrophage and DC progenitors (25), to regulate GM-CSF-induced DC differentiation (31).

We confirmed that the development of the principal DC subtype CD11b^{int}MHC^{int}Ly-6C^{neg} was primarily impaired in the absence of ERα signaling, whereas the development of CD11c$^+$ cells expressing high levels of CD11b and Ly-6C and low levels of MHC-II was spared. This estrogen insensitive subset might correspond to a monocyte/macrophage-like population usually present at low frequency in WT BMDC cultures (15, 32). Indeed, we observed a 2- to 3-fold increase in macrophage-like cells in ER$\alpha^{-/-}$ DC cultures by cytological staining (data not shown). This observation correlated with an increased frequency of cells expressing high levels of TLR4-MD2 active complexes and CD11b in ER$\alpha^{-/-}$ DCs or in estrogen-deprived WT BMDC, in agreement with previous work (20). This could explain the higher propensity of ER$\alpha^{-/-}$ DCs to produce cytokines upon LPS stimulation as both TLR4 and the β_2 integrin CD11b have been shown to act in concert to positively regulate MyD88-dependent LPS signaling in macrophages (33, 34). The commitment of myeloid progenitors to DCs vs macrophages could be therefore differentially regulated by E_2 signaling under GM-CSF-induced differentiation. It has been proposed that high PU.1 activity could favor DCs at the expenses of macrophage fate through the negative regulation of the macrophage-specific transcription factor Maf-B (35). ERα signaling during DC development could therefore regulate the balance between PU.1, Maf-B, or others transcription factors (30), thereby modulating DC differentiation.

The capacity of DCs to respond to T cell-dependent signals is critical to initiate adaptive immune responses and drive Ag-specific CD4$^+$ T cell activation and differentiation through CD40-dependent production of polarizing cytokines such as IL-12, IL-23, and IL-6 (36). Our data clearly showed that DCs generated in the absence of E_2 or ERα signaling exhibited an impaired capacity to activate naive CD4$^+$ T cells as compared with DCs generated in the presence of E_2. The low level of MHC-II and CD86 costimulatory molecule expressed by the main CD11b^{high} DC subsets from ER$\alpha^{-/-}$ or E_2-deprived WT cultures can partly account for their inability to prime CD4$^+$ T cell proliferation. Additionally, we found that E_2-dependent ERα activation during in vitro DC differentiation enhances CD40-dependent production of IL-12 and IL-6, two important polarizing cytokines that drive expansion of naive CD4$^+$ T cells to the Th1 or Th17 pathway, respectively (36). By contrast, E_2 treatment on already differentiated DCs during stimulation with TLR or CD40 ligands did not significantly modify cytokine secretion profiles. Thus, despite numerous studies showing that estrogens could inhibit NF-κB and suppress proinflammatory cytokine expression in myeloid cells in vitro (18, 19, 37), we were unable to document any significant inhibitory effect of E_2 on either TLR- or CD40-dependent cytokine production by DCs. Thus, differential cytokine production between DCs that developed in the absence or presence of E_2 signaling is imprinted during GM-CSF-induced differentiation and therefore reflects an E_2 effect on precursors or developing DCs rather than on already differentiated cells.

Generation of conventional GM-CSF-induced BMDC is usually performed in culture medium exhibiting an estrogenic activity (estrogens present in standard FCS but also the pH indicator phenol red). Interestingly, addition of a wide dose range of E_2 from 0.1 to 10 nM in steroid-free medium could restore DC development and in conventional medium could further increase CD40-dependent cytokine production. Concentrations of E_2 between 0.1 and 1 nM correspond to physiological levels of E_2 found in adult female mice during diestrus (20–35 pg/ml) and estrus (100–200 pg/ml), respectively (38), suggesting that low levels of E_2 could potentially modulate immune responses in vivo. Indeed, we have shown that administration of E_2 in castrated C57BL/6 (B6) mice resulted in a marked up-regulation of Ag-specific CD4 T cell responses and in the selective development of IFN-γ-producing cells through ERα signaling in hemopoietic cells (39). Interestingly, E_2 has been also shown to selectively enhance IFN-γ-production by NKT cells in vivo (40). Furthermore, E_2 treatment was also shown to enhance the susceptibility to experimental autoimmune myasthenia gravis, a Th1-dependent B cell-mediated autoimmune disease (41). However, whether this effect of E_2 in vivo is due to a direct modulation of DC development and/or function remains to be investigated. Understanding further the impact of ER signaling on DC biology may therefore provide new insights into the mechanisms by which sex-linked factors affect immunity and susceptibility to autoimmune diseases in women.

Acknowledgments

C. Coureau and the staff of the IFR30 animal facility are acknowledged for skillful technical assistance. We thank Dr. P. Hwu (National Cancer Institute, Bethesda, MD) and Dr. C. Reis e Sousa (Cancer Research U.K., London) for providing the CD40L-transfected fibroblasts.

Disclosures

The authors have no financial conflicts of interest.

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