

# 17 $\beta$ -Estradiol Alters the Activity of Conventional and IFN-Producing Killer Dendritic Cells<sup>1</sup>

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Estrogens increase aspects of innate immunity and contribute to sex differences in the prevalence of autoimmune diseases and in response to infection. The goal of the present study was to assess whether exposure to 17 $\beta$ -estradiol (E2) affects the development and function of bone marrow-derived dendritic cells and to determine whether similar changes are observed in CD11c<sup>+</sup> splenocytes exposed to E2 in vivo. E2 facilitated the differentiation of BM precursor cells into functional CD11c<sup>+</sup>CD11b<sup>+</sup>MHC class II<sup>+</sup> dendritic cells (DCs) with increased expression of the costimulatory molecules CD40 and CD86. Exposure of bone marrow-derived dendritic cells to E2 also enhanced production of IL-12 in response to the TLR ligands, CpG and LPS. In contrast, CD11c<sup>+</sup> cells isolated from the spleens of female C57BL/6 mice that were intact, ovariectomized, or ovariectomized with E2 replacement exhibited no differences in the number or activity of CD11c<sup>+</sup>CD11b<sup>+</sup>MHC class II<sup>+</sup> DCs. The presence of E2 in vivo, however, increased the number of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells and reduced numbers of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>high</sup> cells, a surface phenotype for IFN-producing killer DCs (IKDCs). Ultrastructural analysis demonstrated that CD11c<sup>+</sup>NK1.1<sup>+</sup> populations were comprised of cells that had the appearance of both DCs and IKDCs. CD11c<sup>+</sup> splenocytes isolated from animals with supplemental E2 produced more IFN- $\gamma$  in response to IL-12 and IL-18. These data illustrate that E2 has differential effects on the development and function of DCs and IKDCs and provide evidence that E2 may strengthen innate immunity by enhancing IFN- $\gamma$  production by CD11c<sup>+</sup> cells. *The Journal of Immunology*, 2008, 180: 1423–1431.

Sex-based differences in susceptibility to both autoimmune and infectious diseases are widely reported (1). Approximately 80% of all cases of autoimmunity in the U.S. are women, with differences between the sexes being most pronounced for Sjogren's syndrome, systemic lupus erythematosus, thyroid disease (Hashimoto's thyroiditis and Graves' disease), scleroderma, and myasthenia gravis (2, 3). Although females are more likely to develop autoimmune diseases, the prevalence and intensity of viral, bacterial, and parasitic infections are typically reduced in females compared with males (4). Sex differences in susceptibility to autoimmune and infectious diseases may reflect an immunological dimorphism, in which females often mount more robust immune responses than males (4–6). The prevailing hypothesis for heightened immunological responsiveness in females compared with males is that sex hormones, in particular testosterone and 17 $\beta$ -estradiol (E2),<sup>3</sup> directly influence immune function.

Estrogens are lipophilic hormones that exert their biological effects by binding to intracellular receptors, specifically estrogen receptor (ER)  $\alpha$  and ER $\beta$ . Both receptor subtypes are present in the thymus and spleen as well as in circulating lymphocytes, dendritic cells (DCs), NK cells, and macrophages (7–10). The binding of E2 to the ER is sufficient to alter the effector functioning of immune cells. For example, exposure to E2 enhances cytotoxicity and production of IFN- $\gamma$  by NK cells (11, 12); a process likely mediated by the presence of estrogen response elements (ERE) in the promoter region of the *ifng* gene (13). E2 also facilitates the differentiation of bone marrow (BM) progenitor cells into functional CD11c<sup>+</sup> DCs in vitro (14); enhances the synthesis of inducible NO synthase and proinflammatory cytokines, including IL-1, IL-6, and TNF, by macrophages (15, 16); and augments the expansion of CD4<sup>+</sup>CD25<sup>+</sup> T cells in mice (17). Although there are reports of E2 suppressing proinflammatory responses, including production of IL-6 by macrophages (18–20), generally, estrogens enhance both cell-mediated and humoral immune responses (21, 22). These effects, however, can be dependent on the dose of E2, the timing of estrogen treatment, and the cell population examined (19, 20).

A wide array of immune effector mechanisms mediates dimorphic autoimmune and infectious disease outcomes; thus, sex differences in disease susceptibility cannot be attributed to one particular arm of the immune system that may be differentially regulated in females than in males. Much of the work investigating sex-based differences in autoimmune and infectious diseases has focused on end-point effector mechanisms of adaptive immunity, such as Ab production, cell-mediated cytotoxicity, and cytokine production (23–26). Whether sex-associated susceptibility to autoimmune and infectious diseases also reflects differences in the activity of the innate immune system is less well characterized (27). DCs serve as a critical link between innate and adaptive immunity and are essential for activation and regulation of immune responses against infection (28, 29). In mice, subsets of splenic DCs

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<sup>3</sup> Abbreviations used in this paper: E2, 17 $\beta$ -estradiol; ER, estrogen receptor; ERE, estrogen response element; DC, dendritic cell; BM, bone marrow; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; IKDC, interferon-producing killer dendritic cell; ovx, ovariectomized; sham, sham surgery; WT, wild type; MHCII, MHC class II; IMDM, Iscove's modified Dulbecco's medium.

can be differentiated based on surface marker expression and cytokine production: 1) conventional DCs (cDCs) are CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> or CD11c<sup>+</sup>CD11b<sup>+</sup> and produce IL-12; 2) plasmacytoid DCs (pDCs) are CD11c<sup>dim</sup>B220<sup>+</sup>Gr1<sup>+</sup> and produce primarily type I IFNs following stimulation; and 3) IFN-producing killer DCs (IKDCs) are CD11c<sup>+</sup>MHCII<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>high</sup> and produce type I IFNs, IL-12, and IFN- $\gamma$ , depending on the stimulus (30).

To date, studies examining the effects of E2 on DCs have primarily been restricted to in vitro stimulation of BM-derived precursors, with a focus on cDC populations (14, 31). In the work presented in this study, we expand these observations and demonstrate that E2 differentially affects the number, phenotype, and activity of cDCs and IKDCs. Furthermore, we establish that E2 has distinct effects on BM-derived precursors stimulated with E2 in vitro as compared with CD11c<sup>+</sup> splenocytes exposed to E2 in vivo. Specifically, we observed that E2 promotes the differentiation and activity of cDCs from BM-derived precursors and augments the differentiation of CD11c<sup>+</sup> splenocytes into an alternative DC phenotype that morphologically resemble IKDCs, express CD49b and NK1.1, and synthesize high amounts of IFN- $\gamma$ . These results suggest that E2 plays a significant role in enhancing innate immune activation and may provide insight into the mechanism underlying sex-based differences in disease outcomes.

## Materials and Methods

### Animals

Adult (>60 days of age) female ( $n = 400$ ; 5–10/group/replication/experiment) C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD) and Esr1<sup>-/-</sup> females on a C57BL/6 background ( $n = 5$ ) were purchased from Taconic Farms. All animals were housed five per cage in a microisolator room and maintained on a constant light:dark 16:8 cycle. Food and sterile tap water were available ad lib. The Johns Hopkins Animal Care and Use Committee (protocol # MO04H532) approved all procedures described.

### Ovariectomy and hormone replacement

Animals were assigned to remain intact or be bilaterally ovariectomized (ovx). Mice were bilaterally ovx under ketamine (80 mg/kg body mass)–xylazine (8 mg/kg body mass) anesthesia (Phoenix Pharmaceutical) and given 2 wk to recover from surgery. Intact females received sham surgeries (sham). After recovery, females assigned to have E2 replaced, were each s.c. implanted with a 10 mm length Silastic capsule (i.d. = 1.02 mm, o.d. = 2.16 mm) filled with either 2.5 mm or 5 mm (5 mm of E2 was used in all experiments, except where noted; ovx+E2) of E2 (Sigma-Aldrich) and sealed with Silastic adhesive to form a controlled-release capsule as described previously (32). The remaining ovx females as well as sham females were each implanted with an empty Silastic capsule of equal length. The 5-mm dose of E2 was based on a previous report that this amount is sufficient to maintain physiological E2 concentrations (i.e., 100–200 pg/ml) in female mice for 14 days (33). Estradiol-containing and control Silastic capsules were incubated in sterile saline (37°C) for 24 h before s.c. implantation in the midscapula region. All animals were processed 2 wk after implanting capsules. Serum E2 concentrations were assayed using a standard ether extraction and a rodent E2 EIA kit, optimized in our laboratory (Cayman Chemicals). Uterine horns were removed and weighed as an additional bioassay for E2 concentrations.

### Generation of DCs from BM cells

BM cells were isolated from the femurs and tibiae of adult female C57BL/6 mice and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% culture supernatant from GM-CSF-producing J558 cells (14). Because phenol red is weakly estrogenic (34), we conducted preliminary experiments to compare the phenotype and activity of BMDCs that were incubated in standard culture media that contained phenol red (IMDM (Invitrogen Life Technology) plus 10% filtered FBS (Sigma-Aldrich) with cells incubated in estrogen-deficient media that contained phenol red-free IMDM (Invitrogen Life Technology) supplemented with 10% charcoal-dextran-treated FBS (HyClone). As reported previously (14), standard culture media had a significant effect on BMDCs (data not shown); thus, all in vitro experiments were conducted using estrogen-deficient media only. Total BM cells were suspended at  $1 \times 10^6$  cells per ml

in estrogen-deficient medium in 12-well plates. Estrogen-deficient medium contained phenol red-free IMDM, 10% charcoal-dextran-treated FCS, L-glutamine, penicillin/streptomycin, gentamicin, and 2-ME (Invitrogen Life Technologies and Sigma-Aldrich). On days 3 and 6, half of the culture medium was removed and replaced with fresh estrogen-deficient medium containing GM-CSF. Estradiol (Steraloids) and/or ICI 182,780 (Tocris) were added at the beginning of the culture and were replenished with the medium on days 3 and 6. DCs were harvested on day 9.

### Cell isolation and flow cytometry

Spleens were dissected and splenocytes were isolated by mincing and digesting whole tissue in 2 mg/ml collagenase D (Roche). Tissues were homogenized, cells were filtered and washed several times in PBS supplemented with 2 mM EDTA and 2% FCS, and mononuclear cells were isolated by resuspending cells in ACK lysis buffer (Invitrogen Life Technologies). Nonspecific binding was blocked by staining with FcR. Cell samples were stained with CD11c FITC, incubated with anti-FITC microbeads, and processed through LS columns using the quadroMACS magnet (Miltenyi Biotec). Retained cells were collected and viable cells were counted using a hemacytometer and trypan blue exclusion. Cells were then stained for appropriate mouse DC, NK, and IKDC markers. Cells were stained with the following mAbs: anti-mouse CD49b-PE (clone DX5), NK1.1-APC (clone PK136), CD11b-PE (clone M1/70), H2D<sup>b</sup>-PE (clone KH95), CD8 $\alpha$ -PE (Ly-2; clone 53-6.7), CD45R-APC (B220; clone RA3-6B2), Ly-6G-PE (Gr1; clone RB6-8C5), CD40-PE (clone IC10), NKG2D-PE (clone C7), and CD122-PE (clone 5H4) (BD Biosciences; eBiosciences).

### Cell activation and cytokine production

Cells were stimulated with 5 ng/ml rIL-12 (eBiosciences), 5 ng/ml rIL-18 (MBL), 1  $\mu$ g/ml LPS (Sigma-Aldrich), 1  $\mu$ g/ml poly I:C (Invivogen), 1  $\mu$ g/ml CpG (Integrated DNA Technologies), or 1  $\mu$ g/ml a control oligonucleotide (Integrated DNA Technologies) for 24 h. Cytokine concentrations were assayed by ELISA using the manufacturer's protocols for IFN- $\gamma$ , IL-12p40, IL-12p70 (BD Pharmingen), and IFN- $\alpha$  (PBL Biomedical Laboratories).

### NK cell lysis

Viable CD11c<sup>+</sup> cells were adjusted to  $1 \times 10^6$  cells/ml and incubated with <sup>51</sup>Cr-labeled YAC-1 target cells (American Type Culture Collection) for 4 h at 37°C at E:T ratios ranging from 100:1 to 12.5:1. After the 4 h incubation, <sup>51</sup>Cr released from target cells was counted using a  $\gamma$  counter and lysis was calculated as: ((measured <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release)/(maximum <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release))  $\times$  100. Maximum release was determined based on acid-lysed target cells and spontaneous release was determined by incubating target cells in the absence of effector cells. The average percent cytotoxicity for each set of duplicates was used in statistical analyses.

### Microscopy

CD11c<sup>+</sup> splenocytes were isolated as described above and sorted into CD49b<sup>+</sup>NK1.1<sup>high</sup> and CD49b<sup>+</sup>NK1.1<sup>low</sup> populations on a FACS Aria (BD Biosciences). For microscopy, sorted cells were fixed with 2% glutaraldehyde for 2 h at 24°C followed by postfixation with 2% osmium tetroxide for 2 h at 24°C. The cells were embedded in Epon 812, sectioned at 2  $\mu$ m, stained with toluidin blue, and examined by light microscopy using a Nikon Eclipse E800 light microscope (Nikon). Images were acquired at 60 $\times$  using a SPOT RT CCD imager and software (Diagnostic Instruments).

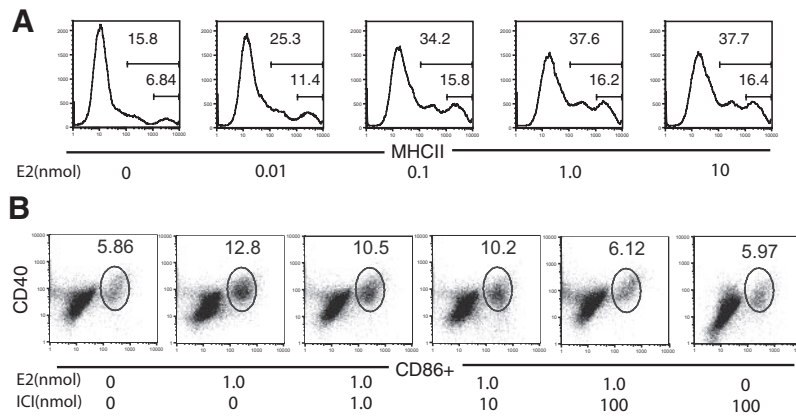
### Statistical analyses

The effects of E2 on all dependent measures were analyzed using one- or two-way ANOVAs with treatment group and/or TLR ligand as the independent variable(s). Significant interactions were further analyzed using the Tukey method for pairwise multiple comparisons. Comparisons between Esr1<sup>-/-</sup> and wild-type (WT) females were conducted using Student's *t* tests. If the data sets violated the assumptions of a normal distribution, then nonparametric statistics were used. Mean differences were considered statistically significant if  $p < 0.05$ .

## Results

### 17 $\beta$ -estradiol enhances the differentiation of naive BM-derived DCs

In vitro exposure to physiological concentrations of E2 facilitates differentiation of BM precursor cells into functional CD11c<sup>+</sup> DCs with increased levels of MHC class II (MHCII), CD80, and CD86



**FIGURE 1.**  $17\beta$ -estradiol (E2) facilitates differentiation of DCs from bone marrow (BM). Bone marrow cells were cultured in estrogen-deficient medium with GM-CSF for 8 days in the absence or presence of the indicated concentrations of E2. Nonadherent cells were harvested and assessed for surface markers characteristic of mature cDCs by FACS. Plots represent the expression of I-A<sup>b</sup> on CD11c<sup>+</sup> cells. Values represent the percentage of MHCII<sup>+</sup> (top) and MHC II<sup>high</sup> (bottom) of the total CD11c<sup>+</sup> cells (A). Bone marrow cells were cultured in estrogen-deficient medium with GM-CSF, E2 (1 nM), and the indicated concentrations of the ER antagonist, ICI 182,780. On day 8, cells were stained for FACS analysis. Plots represent CD40 v. CD86 expression on CD11c<sup>+</sup> cells. Values represent the percentage of CD40<sup>+</sup>CD86<sup>+</sup> of the total CD11c<sup>+</sup> cells (B). Results are representative of three independent experiments.

on their surface (14). Many cells, however, express CD11c, including cDCs, pDCs, IKDCs, and NK cells (35, 36). We confirmed and expanded previous findings by generating CD11c<sup>+</sup> cells from BM precursors, incubating cells with varying concentrations of E2 and/or the ER antagonist, ICI 182,780, and assessing cells for surface markers characteristic of cDCs, pDCs, IKDCs, and NK cells. Exogenous administration of 0.1–10 nM of E2 (i.e., concentrations within the range circulating in adult female mice (37)) facilitated differentiation of BM precursor cells into functional CD11c<sup>+</sup> DCs and increased the expression of MHCII (Fig. 1A), CD11b, CD40, and CD86, but not NK1.1 or CD49b (data not shown). The effect of E2 was mediated by the estrogen receptor because blocking ER $\alpha$  and ER $\beta$  with 100 nM of ICI 182,780 significantly inhibited DC differentiation ( $p < 0.05$ ; Fig. 1B and data not shown). Neither E2 nor ICI 182,780 alone or in combination affected BMDC viability as tested by 7-AAD staining (data not shown). ICI 182,780 alone also did not significantly affect differentiation of BMDCs ( $p < 0.05$ ; Fig. 1B and data not shown). These data illustrate that the *in vitro* effects of E2 on BM-derived CD11c<sup>+</sup> cells are limited to cDCs and are mediated by the ER.

#### *17 $\beta$ -estradiol is required for activation of BMDCs by TLR ligands*

To determine the effect of E2 on the ability of DCs to initiate an innate immune response, BM-derived cells differentiated in the presence of either no E2 or 1 nM of E2 (i.e., the median dose from Experiment 1) and were exposed to the TLR ligands LPS, poly I:C, CpG, media alone, or a control ODN. Cells were assessed for surface markers characteristic of mature cDCs and supernatants were collected to measure cytokine concentrations. E2 enhanced the expression of MHCII, CD40, and CD86 on CD11c<sup>+</sup> BMDCs as compared with cells not treated with E2, regardless of the TLR agonist used ( $p < 0.05$ ; Figs. 2, A and B). Strikingly, BM-derived DCs differentiated in the absence of E2 showed no activation in response to stimulation with any of the TLR ligands. Among E2-treated BMDCs, exposure to CpG and LPS, but not poly I:C or the control ODN, further enhanced the expression of MHCII, CD40, and CD86 as compared with exposure to media alone ( $p < 0.05$ , Fig. 2B). Treatment of BMDCs with E2 increased the production of IL-12p40 in response to CpG and LPS, but not poly I:C or the control ODN ( $p < 0.05$ ; Fig. 2C). Exposure to E2 also facilitated

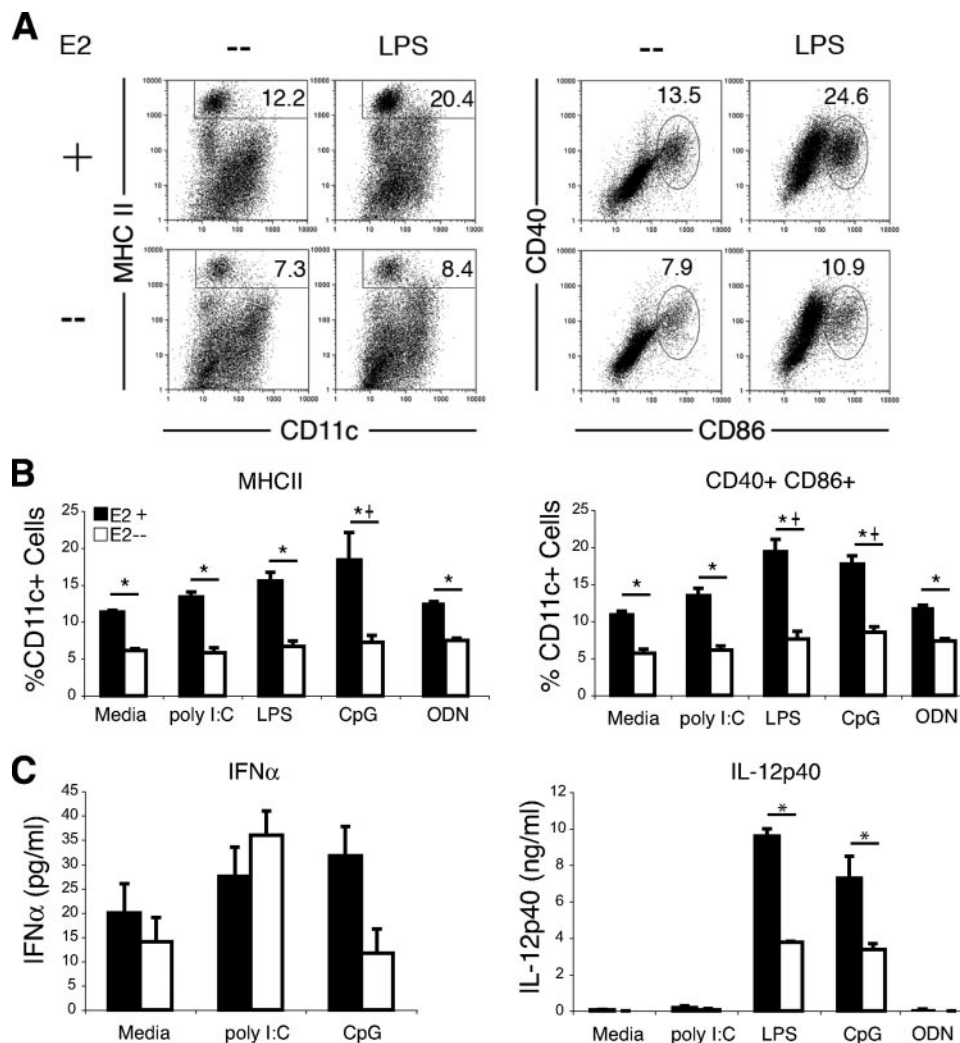
production of IL-12p70 by BMDCs in response to LPS (E2:  $3.10 \pm 0.41$  ng/ml vs media:  $1.54 \pm 0.01$  ng/ml;  $p < 0.05$ ), but not CpG, poly I:C, or the control ODN (data not shown). E2 treatment tended to increase the synthesis of IFN- $\alpha$  in response to CpG, but this difference did not reach statistical significance ( $p > 0.05$ ; Fig. 2C).

Previous studies illustrate that BMDCs can produce IFN- $\gamma$  following stimulation with IL-12 and IL-18 (38, 39). In addition to being responsive to classic TLR ligands, we sought to determine whether differentiated DCs from BM precursors were responsive to IL-12 and IL-18 and could produce IFN- $\gamma$  in the presence of E2. BMDCs were differentiated as described above, exposed to either E2 or media alone, and activated with rIL-12 and rIL-18. BMDCs stimulated with IL-12 and IL-18 exhibited no changes in the expression of MHCII, CD40, and CD86 or the synthesis of IFN- $\gamma$ , regardless of E2 exposure ( $p > 0.05$ ; data not shown).

#### *17 $\beta$ -estradiol increases numbers of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells in the spleen*

The data presented in this study and by others (14) illustrate that E2 impacts the *in vitro* differentiation of BM-derived precursors into functional cDCs. The effect of E2 on the phenotype and function of DCs in peripheral lymphoid organs, however, has not been addressed. To test whether E2 alters the number, surface phenotype, or activity of splenic DCs, we conducted a series of *in vivo* studies using intact/sham females, ovx females, and ovx females with E2 replacement (ovx+E2). Serum levels of E2 were significantly different among the three groups of females, in which concentrations were elevated in ovx+E2 females and reduced in ovx females relative to sham female mice ( $p < 0.05$ ; Fig. 3A). Exogenous replacement of E2 resulted in circulating estradiol concentrations that were within the physiological range (100–200 pg/ml) for female mice in estrus (37). Uterine horn mass was measured as a bioassay and was consistent with E2 concentrations, in which ovx+E2 and sham females had heavier uterine horns than ovx female mice ( $p < 0.05$ ; Fig. 3B).

CD11c<sup>+</sup> splenocytes from C57BL/6 mice are a heterogeneous population of cells comprised of multiple subsets of DCs, including cDCs, pDC, and IKDCs, all of which are classified based on their surface marker profiles (30, 36, 40). To examine the effects of E2 on the absolute numbers and percentages of DCs in the spleen, CD11c<sup>+</sup> splenocytes were isolated and stained for MHCII, CD11b,



**FIGURE 2.** E2 enhances the activation of CD11c<sup>+</sup> BMDCs by TLR ligands. BMDCs were cultured in estrogen-deficient medium for 8 days and stimulated with TLR agonists or control ODN for the last 24 h of culture. Nonadherent cells were harvested and stained for FACS analysis. Representative plots of CD11c v. MHC II and CD40 v. CD86 (gated on CD11c<sup>+</sup> cells) are shown (A). Values represent the percentage of MHCII<sup>high</sup> and CD40<sup>+</sup>CD86<sup>+</sup> cells. Summary of the proportion of CD11c<sup>+</sup> BMDCs ( $\pm$ SEM) that expressed MHCII<sup>high</sup>, CD40, and CD86 following culture with (E2<sup>+</sup>) or without (E2<sup>-</sup>) 1 nM E2 and activation with the indicated TLR ligands (B). Mean production of IFN- $\alpha$  and IL-12p40 ( $\pm$ SEM) by BMDCs exposed to either 1 nM of E2 or media alone and activated with the designated TLR ligands (C). CD11c<sup>+</sup> BMDCs were generated by incubating cells with GM-CSF and either 1 nM of E2 (E2<sup>+</sup>) or media alone (E2<sup>-</sup>). During the last 24 h of the 8-day incubation, cells were activated with LPS, poly I:C, CpG, control ODN, or media alone. On day 9, cells were assessed for surface markers characteristic of mature cDCs by FACS and supernatants were collected to measure cytokine concentrations by ELISA. Results are representative of three independent experiments. An asterisk (\*) indicates that E2 enhances responses relative to media alone, a dagger (†) indicates that among E2-treated BMDCs, TLR ligands enhanced responses relative to media alone,  $p < 0.05$ .

B220, CD8 $\alpha$ , CD49b, and the costimulatory molecules, CD40 and CD86. In contrast to the effects on BMDCs, E2 did not significantly affect the absolute number of CD11c<sup>+</sup> cells isolated from the spleen or the percentage of CD11c<sup>+</sup> cells determined to be cDCs based on the high expression of CD11c and MHCII ( $p > 0.05$ , data not shown). Neither increasing nor decreasing concentrations of E2 in vivo altered the percentage of CD11c<sup>+</sup>CD11b<sup>+</sup> cDCs, the percentage of CD8 $\alpha$ <sup>+</sup> cDCs, the percentage of B220<sup>+</sup> pDCs, or the expression of the costimulatory molecules CD40 and CD86 on any of the DC subsets ( $p > 0.05$ ; Fig. 3C and data not shown).

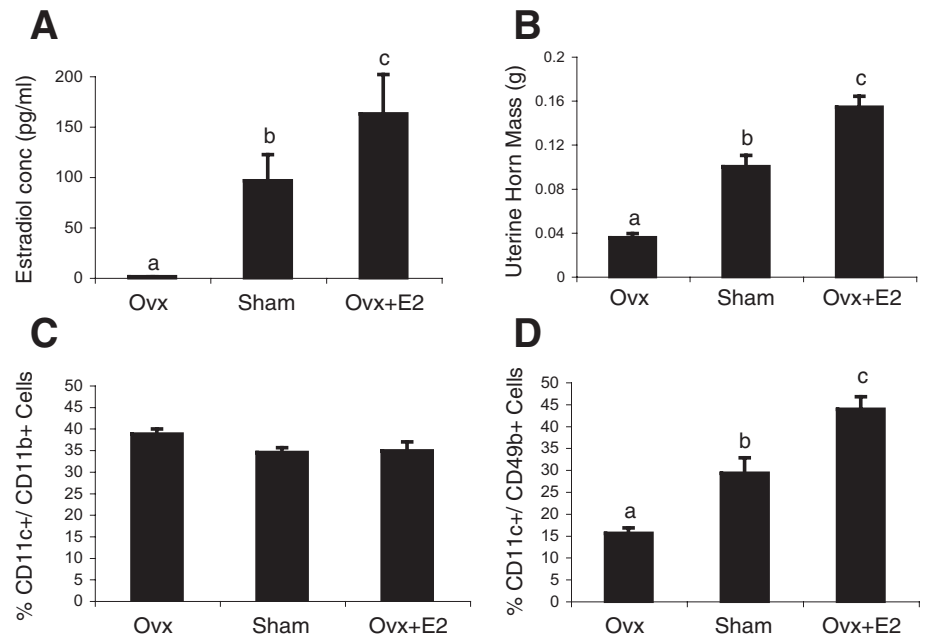
There is overlapping expression of surface markers among DCs, NK cells, and IKDCs that can make the precise characterization of these cell populations complicated. Both IKDCs and NK cells express CD11c, CD49b, and NK1.1; thus, we sought to examine whether these cells were responsive to E2. Removal of circulating E2 via ovariectomy decreased, whereas sustained exposure to E2 dramati-

cally increased the percentage of CD11c<sup>+</sup>CD49b<sup>+</sup> cells in the spleen as compared with the sham controls ( $p < 0.05$ ; Fig. 3D).

To establish whether E2 alters the number of IKDCs in the spleen, we enriched for CD11c<sup>+</sup> cells and stained for both CD49b (i.e., integrin subunit  $\alpha 2$ ) and NK1.1 (i.e., NK receptor P1C). By gating on live CD11c<sup>+</sup> cells, we observed that sustained exposure to E2 reproducibly increased the number of CD49b<sup>+</sup>NK1.1<sup>low</sup> cells in the spleen as compared with sham and ovx female mice ( $p < 0.05$ ; Fig. 4, A and C). In contrast, the absence of E2 in ovx females increased the proportion of splenic CD49b<sup>+</sup>NK1.1<sup>high</sup> cells as compared with ovx+E2 and sham female mice ( $p > 0.05$ ; Fig. 4, B and C).

To further illustrate the dependence of these changes on biologically relevant concentrations of E2, we implanted ovx females with a lower dose of E2 (i.e., 2.5 mm of E2 in 10 mm capsules). Similar to ovx females implanted with a higher dose (i.e., 5 mm of

**FIGURE 3.** E2 differentially affects subpopulations of CD11c<sup>+</sup> cells in the spleen. Serum, uterine horns, and spleens were collected from intact (sham), ovariectomized (ovx), and ovx females that had E2 replaced exogenously (ovx+E2). Circulating E2 concentrations were measured by ELISA ( $\pm$ SEM) (A) and uterine horns were weighed ( $\pm$ SEM) (B). Whole splenocytes were isolated, enriched for CD11c<sup>+</sup> cells, and stained for CD11b (C) or CD49b (D). FACS analysis was performed by gating on CD11c<sup>+</sup> cells. Bars with different letters indicate significant differences across groups ( $n = 10$ /group),  $p < 0.05$ .

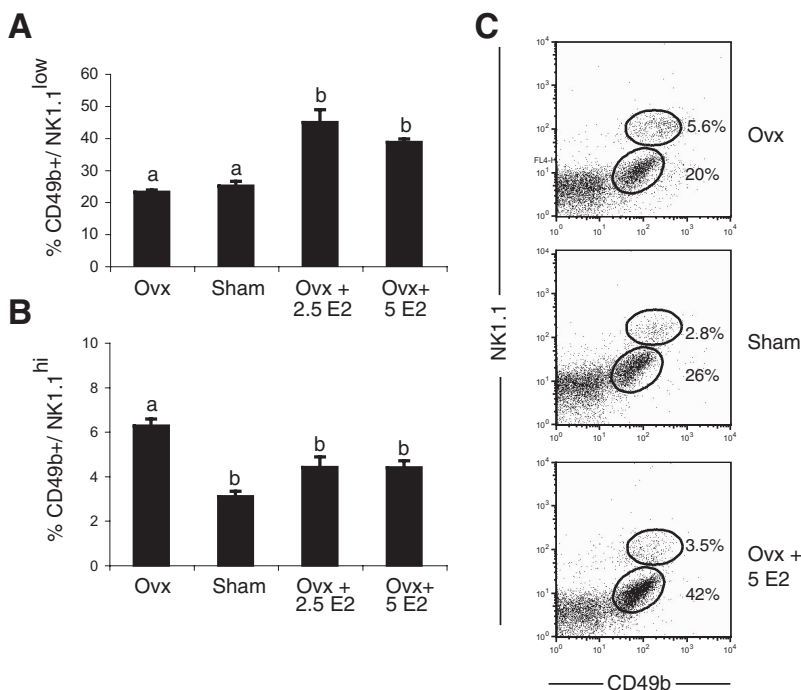


E2 in 10 mm capsules), ovx females exposed to a low dose of E2 also had elevated numbers of splenic CD49b<sup>+</sup>NK1.1<sup>low</sup> cells and lower numbers of CD49b<sup>+</sup>NK1.1<sup>high</sup> cells as compared with ovx female mice ( $p < 0.05$  in each case; Fig. 4).

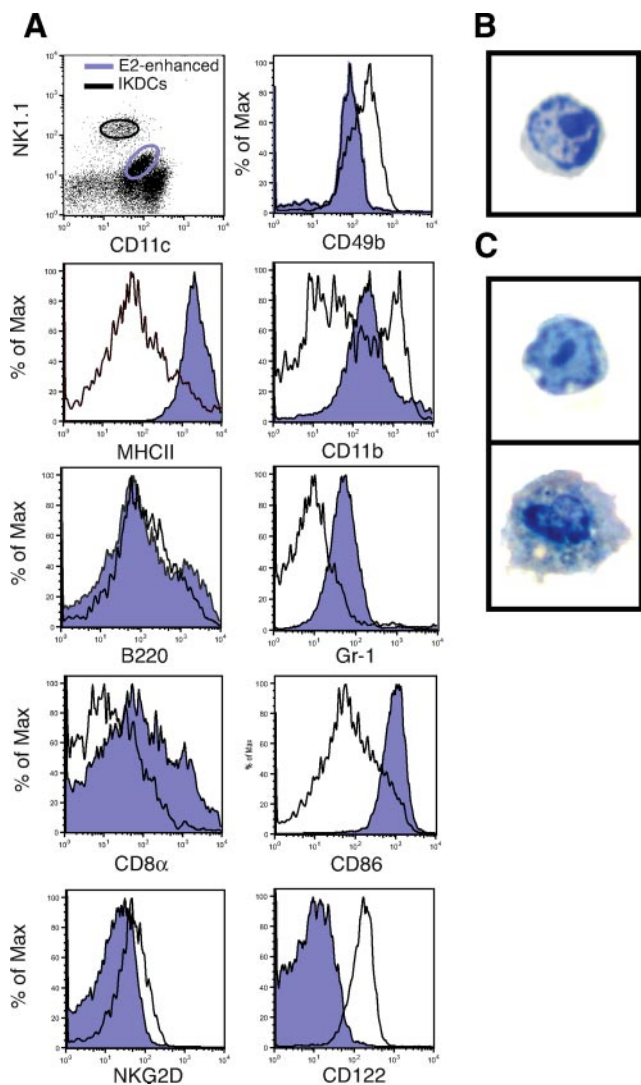
*17 $\beta$ -estradiol reduces numbers of splenic IKDCs*

IFN-producing killer DCs are cells with surface expression characteristics of both DCs and NK cells (36). To further characterize the cell populations influenced by E2 as cDCs, IKDCs, or cells that share both cDC and IKDC markers, we stained and evaluated CD11c<sup>+</sup> splenocytes from sham, ovx, and ovx+E2 mice for surface levels of MHCII, B220, CD8 $\alpha$ , CD11b, CD11c, CD40, CD86, Gr-1, CD49b, NK1.1, CD122, and NKG2D (36). The CD49b<sup>+</sup>NK1.1<sup>high</sup> cells, which were reduced in number by sus-

tained E2 exposure, expressed appropriate levels of all the markers reportedly expressed on IKDCs, including CD11c, NK1.1, CD49b, CD122, MHCII, and B220 (Fig. 5A) (36). The CD49b<sup>+</sup>NK1.1<sup>low</sup> population of CD11c<sup>+</sup> cells, which was increased in number by exposure to E2, did not express the NK cell markers CD122 or NKG2D, but did express MHCII, CD11b, B220, CD8 $\alpha$ , Gr-1, and the costimulatory molecules CD40 and CD86 (Fig. 5A), all of which are markers used to categorize DC subsets (30, 40). The CD49b<sup>+</sup>NK1.1<sup>high</sup> cells had the morphological appearance of IKDCs, including large distinct nuclei and a very small cytoplasm (Fig. 5B) (36). Conversely, the CD49b<sup>+</sup>NK1.1<sup>low</sup> population was comprised of two predominant phenotypes: 1) cells with distinct nuclei and a large flowing cytoplasm, characteristic of cDCs (41); and 2) cells with distinct nuclei and a very small cytoplasm, typical



**FIGURE 4.** E2 increases numbers of CD11c<sup>+</sup> CD49b<sup>+</sup>NK1.1<sup>low</sup>, but reduces numbers of CD11c<sup>+</sup> CD49b<sup>+</sup>NK1.1<sup>high</sup> cells ( $\pm$ SEM) in the spleen. Whole splenocytes were isolated from intact (sham), ovariectomized (ovx), and ovx females that had E2 replaced at low (2.5 mm) and high (5 mm) doses, enriched for CD11c, and stained for both CD49b (i.e., integrin subunit  $\alpha$ 2) and NK1.1 (i.e., NK receptor PIC). Using multiparameter FACS analyses, we gated on live CD11c<sup>+</sup> cells and represent data as the proportion of CD11c<sup>+</sup> cells that expressed CD49b and NK1.1<sup>low/high</sup> (A and B). Representative dot plots are included (C). Bars with different letters indicate significant differences across groups; bars that share letters are not significantly different from each other ( $n = 10$ /group),  $p < 0.05$ .

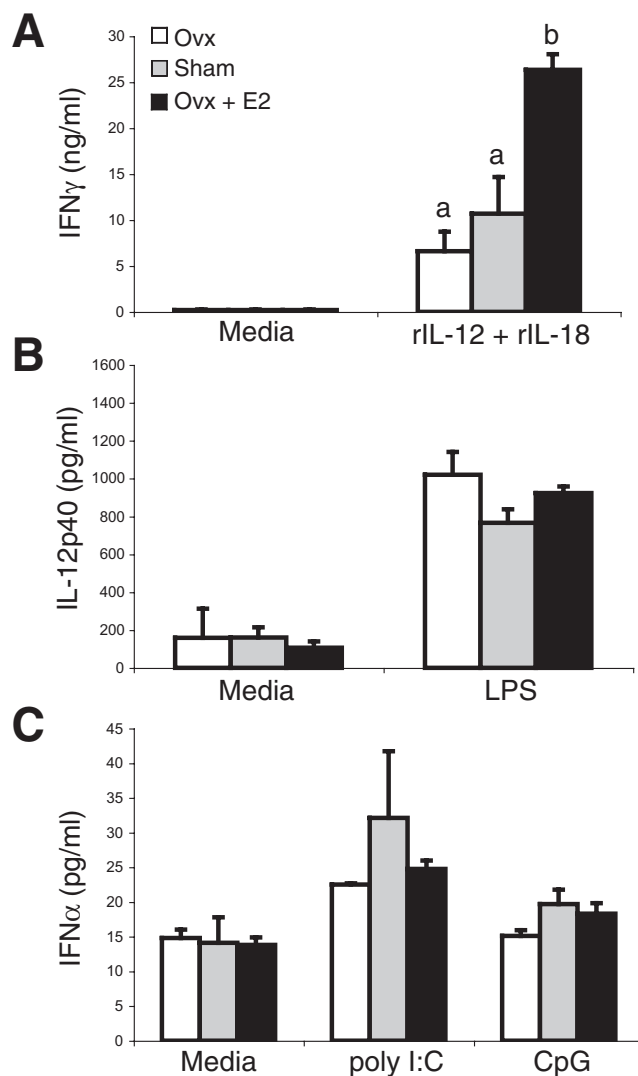


**FIGURE 5.**  $CD11c^+CD49b^+NK1.1^{high}$  cells have phenotypic and morphological characteristics of IKDCs. Whole splenocytes were isolated from the spleens of sham, ovx, and ovx+E2 female C57BL/6 mice, enriched for  $CD11c^+$ , and stained for the following surface markers:  $CD49b$ ,  $CD122$ ,  $NKG2D$ ,  $Gr-1$ ,  $MHCII$ ,  $CD11b$ ,  $B220$ ,  $CD8\alpha$ , and  $CD86$  (A). Morphological analysis using thick section microscopy illustrates the appearance of  $NK1.1^{high}$  (i.e., IKDCs) (B) and  $NK1.1^{low}$  (i.e., cDCs and IKDCs) (C) cells.

of pDCs and IKDCs (Fig. 5C) (36). These data suggested that estrogen-responsive  $CD49b^+NK1.1^{low}$  cells are DCs that share many of the classic features of DC subsets, including IKDCs.

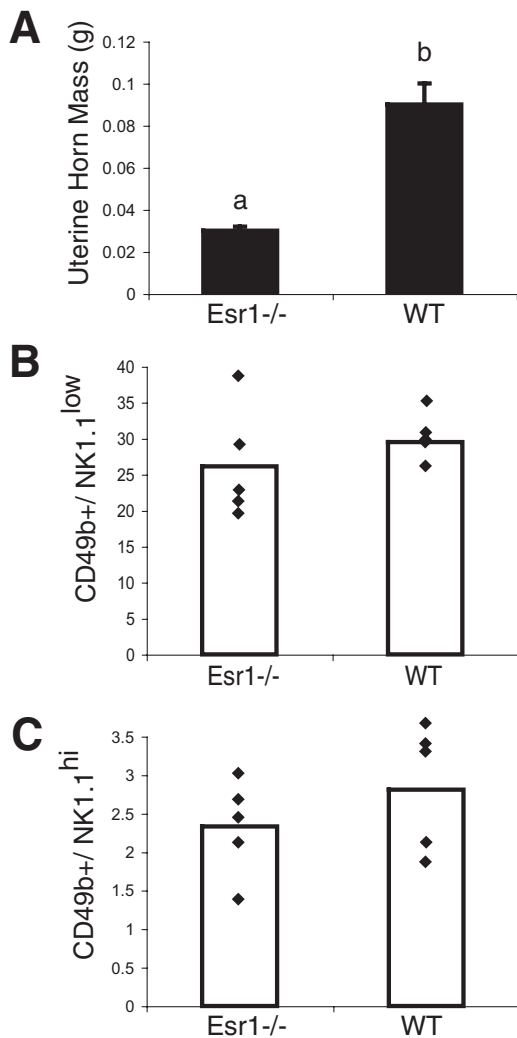
#### *17 $\beta$ -estradiol increases IFN- $\gamma$ production by $CD11c^+$ splenocytes*

Although IKDCs and NK cells comprise only a small percentage of the  $CD11c^+$  cells in the spleen, they are both potent producers of IFN- $\gamma$  (35, 36). Therefore, we hypothesized that the effects of E2 on  $CD49b^+NK1.1^+$  populations may result in functional changes in IFN- $\gamma$  production by  $CD11c^+$  cells.  $CD11c^+$  splenocytes were isolated from ovx, sham, and ovx+E2 females and stimulated with either rIL-12 and rIL-18 or media alone (42). When stimulated with rIL-12 and rIL-18,  $CD11c^+$  splenocytes produced elevated concentrations of IFN- $\gamma$  in the presence of high E2, despite the concomitant decrease in the percentage of  $CD49b^+NK1.1^{high}$  cells ( $p < 0.05$ ; Fig. 6A). These data suggest that the E2-induced enhancement of  $CD49b^+NK1.1^{low}$  cells contribute to increased IFN- $\gamma$  production.



**FIGURE 6.** Production of IFN- $\gamma$  is enhanced by exposure to E2 in vivo. Whole splenocytes were isolated, enriched for  $CD11c^+$  cells, and stimulated with the indicated TLR ligands or rIL-12 and rIL-18 for 24 h. Supernatants were used to measure the synthesis of IFN- $\gamma$  (A), IL-12p40 (B), and IFN- $\alpha$  (C) by ELISA. Bars (mean concentrations  $\pm$  SEM) with different letters indicate significant differences across groups; bars that share letters are not significantly different from each other ( $n = 10$ /group),  $p < 0.05$ .

Because  $CD11c^+$  DCs can produce IL-12 and IFN- $\alpha$ , we assessed the effects of E2 on the production of these cytokines. Splenocytes were isolated from ovx, sham, and ovx+E2 females, enriched for  $CD11c^+$ , and stimulated with LPS, poly I:C, CpG, or media alone. Although  $CD11c^+$  cells produced elevated levels of IL-12p40 following stimulation with LPS ( $p < 0.05$ ), manipulation of E2 did not affect IL-12p40 production ( $p > 0.05$ ; Fig. 6B) and IL12p70 concentrations were below the limits of detection in all treatment groups. To ensure that the lack of effect of E2 on IL-12 production was not the result of functional or phenotypic changes induced during the positive selection procedure,  $CD11c^+$  splenocytes were isolated using a negative selection protocol. Similarly, manipulation of E2 in vivo did not affect production of IL-12p40 in negatively selected  $CD11c^+$  cells ( $p > 0.05$ ; data not shown). Although poly I:C induced higher production of IFN- $\alpha$  by  $CD11c^+$  cells than did CpG, there was no enhancing or inhibitory effect of E2 on IFN- $\alpha$  secretion ( $p > 0.05$ ; Fig. 6C). These data



**FIGURE 7.** Deletion of ER $\alpha$  (Esr1<sup>-/-</sup>) does not significantly affect populations of NK1.1<sup>low</sup> or NK1.1<sup>high</sup> cells in the spleen. Uterine horns ( $\pm$ SEM) from Esr1<sup>-/-</sup> and WT female mice were weighed (A). Whole splenocytes were isolated from the spleens of Esr1<sup>-/-</sup> and WT female mice, enriched for CD11c, and the percentage of CD11c<sup>+</sup> CD49b<sup>+</sup> NK1.1<sup>low</sup> (B) or CD11c<sup>+</sup> CD49b<sup>+</sup> NK1.1<sup>high</sup> (C) cells were determined ( $\pm$ SEM). Bars with different letters indicate significant differences across groups ( $n = 5$ /group),  $p < 0.05$ .

indicate that E2 does not alter the activity of CD11c<sup>+</sup> cell populations that produce either IL-12 or IFN- $\alpha$  (30, 40).

#### CD11c<sup>+</sup> cells do not exhibit cytolytic activity

To further characterize the functional effects of E2 on CD11c<sup>+</sup> splenocytes and to confirm that these CD11c<sup>+</sup> cells were not NK cells, lytic activity was assessed. In the present study, CD11c<sup>+</sup> splenocytes did not engage in significant killing of YAC-1 cells, regardless of hormone treatment (data not shown).

#### The effects of E2 on mature CD11c<sup>+</sup> cells are partially mediated by ER $\alpha$

There are two subtypes of the ER, ER $\alpha$  and ER $\beta$ ; ER $\alpha$ , however, is the receptor subtype that is required for most of the known estrogenic effects (43). Both receptor subtypes are present in the thymus and spleen as well as in circulating lymphocytes, DCs, NK cells, and macrophages (7–10). ER-mediated transcription can be examined using mice deficient in either ER $\alpha$  (Esr1<sup>-/-</sup>) or ER $\beta$  (Esr2<sup>-/-</sup>) (43). To test whether the effects of E2 on CD11c<sup>+</sup>

splenocytes were mediated by signal transduction activity through ER $\alpha$ , splenocytes were isolated from Esr1<sup>-/-</sup> and WT C57BL/6 female mice, enriched for CD11c, and stained for CD49b and NK1.1 markers. Esr1<sup>-/-</sup> females had significantly smaller uterine horns than their WT female counterparts, illustrating that the biological effect of circulating E2 was diminished in these females ( $p < 0.05$ ; Fig. 7A). Despite the effectiveness of the ER $\alpha$  deficit on reproductive development, the absence of ER $\alpha$  did not significantly alter populations of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> or CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>high</sup> cells as compared with WT females ( $p > 0.05$ ; Fig. 7, B and C). There was, however, a tendency for reduced numbers of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells in Esr1<sup>-/-</sup> as compared with WT females (Fig. 7). Synthesis of IFN- $\gamma$  by CD11c<sup>+</sup> cells also did not differ between Esr1<sup>-/-</sup> and WT female mice ( $p > 0.05$ ; data not shown).

## Discussion

Sex-based differences in the prevalence and intensity of autoimmune and infectious diseases are well documented and likely involve the effects of sex steroids, including testosterone and E2, on immune function (1). With regard to innate immunity, E2 has previously been shown to influence the maturation of BMDCs, with preferential development of BMDCs into cDCs with characteristics of Langerhans cells (14, 31), and to facilitate production of IFN- $\gamma$  by NK cells (11). The results of the studies reported in this study confirm that E2 drives preferential development of CD11b<sup>+</sup>CD11c<sup>+</sup> DCs from BM precursors and increases surface expression of MHCII and the costimulatory molecules CD40 and CD86. The presence of E2 did not affect the expression of B220, CD49b, or NK1.1 on BMDCs. BMDCs also were not responsive to IL-12/IL-18 stimulation, further illustrating that the in vitro effects of E2 are limited to cDC populations.

Although differentiation of BMDCs in the presence of E2 enhances Ag presentation to T cells (14), the full extent to which E2 influences the ability of DCs to mediate innate immunity has not been established. In the present study, only BMDCs matured in the presence of E2 were responsive to LPS, CpG, and, to a lesser extent poly I:C, in which the expression of MHCII, CD40, and CD86 as well as IL-12 production was enhanced. BMDCs matured in the absence of E2 were unresponsive to TLR4 and TLR9 agonists, suggesting that the presence of E2 may be required for MyD88-dependent DC activation in response to microbial signals. Interestingly, a putative ERE has been identified in the promoter region of *Myd88* (44).

To investigate E2-dependent changes in mature DC populations in the spleen, we examined the number, phenotype, and activity of CD11c<sup>+</sup> splenocytes from intact females, ovx females, and ovx females with E2 replacement. In contrast to the effects on BMDCs, E2 did not affect the number of CD11c<sup>+</sup> splenocytes isolated or the percentage of those cells expressing classic cDC markers (e.g., CD11b and CD8 $\alpha$ ), pDC markers (e.g., B220), or activation markers (e.g., MHCII, CD40, and CD86) on any of the examined DC subsets. Sustained elevation of E2, however, resulted in a concomitant increase in the proportion of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells and a reduction in the number of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>high</sup> cells in the spleen. In vivo exposure to sustained E2 also increased production of IFN- $\gamma$  by CD11c<sup>+</sup> splenocytes.

IFN-producing killer DCs were distinguished by the expression of CD49b, NK1.1, CD122, MHCII, and B220. Interestingly, the E2-enhanced population of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells was not readily categorized as a DC or IKDC population by surface marker characteristics. The E2-enhanced NK1.1<sup>low</sup> cells expressed an array of DC markers (e.g., CD11b, B220, CD8 $\alpha$ , Gr-1, MHCII, CD40, and CD86), but did not express the definitive NK cell

marker (e.g., CD122) or exhibit cytolytic activity, suggesting that these cells are likely a subset of DCs and not NK cells. Microscopic analysis of these cells also revealed that the E2-enhanced population of CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells had morphological characteristics associated with cDCs, pDCs, and IKDCs.

Because of the overlapping morphological characteristics defining DC, IKDCs, and NK cells, we attempted to characterize the E2-responsive CD11c<sup>+</sup> cells based on their effector function. Previous data reveal that intact female mice treated with additional E2 exogenously have fewer IKDCs in their BM and produce less IFN- $\gamma$  than intact females not treated with E2 (45). In the present study, stimulation of CD11c<sup>+</sup> splenocytes with IL-12 and IL-18 increased production of IFN- $\gamma$  in the presence of sustained E2 in vivo. Recent analyses reveal that IKDCs and NK cells are the predominant CD11c<sup>+</sup> cells that produce IFN- $\gamma$  (46, 47). Because the E2-enhanced CD11c<sup>+</sup> cells do not express CD122 or NKG2D and do not kill target cells, enhanced production of IFN- $\gamma$  likely reflects the effects of E2 on IKDC populations. There was no effect of E2 on IL-12 production in response to LPS or IFN- $\alpha$  in response to either poly I:C or CpG, suggesting that E2 does not affect the classic functions of either cDCs or pDCs in the spleen.

The developmental pathways for DCs and IKDCs are linked to a common population of progenitor cells (45). Consequently, E2 is a critical signal in the maturation process of lymphoid progenitor cells and ultimately determines which end stage cells develop. IFN-producing killer DC development from BM cells is inhibited by E2 (45). In peripheral lymphoid tissues, such as the spleen, CD11c<sup>+</sup>CD49b<sup>+</sup>NK1.1<sup>low</sup> cells may represent cells that are capable of performing DC and IKDC functions. These cells may be expanded to compensate for the inability of IKDCs to mature in the presence of high E2.

Estrogens have their biological effects by binding to either ER $\alpha$  or ER $\beta$ , the hormone-receptor complex then translocates to the nucleus of the cell where the ER can bind to discrete EREs in the regulatory regions of target genes (48). The ligand-receptor complex also can regulate the transcriptional activity of genes that do not contain a classical ERE in the promoter region by recruiting coregulatory proteins that can activate or repress transcription (48). Finally, estrogens can alter the expression of target genes through regulation of other transcriptional factors (e.g., NF- $\kappa$ B) (49). To establish whether E2 has its biological effects through the ERs, the steroidal compound ICI 182,780 (i.e., fulvestrant) was selected for in vitro studies because it is an ER antagonist in all cell types (50). ICI 182,780 not only functionally blocks the ER, but impairs dimerization and nuclear localization of the ER (50). Blocking ER $\alpha$  and ER $\beta$  with ICI 182,780 before administration of E2 in vitro inhibited differentiation of DCs from BM precursor cells as illustrated by the reduced expression of CD11b, MHCII, CD40, and CD86 as compared with cells treated with E2 alone. The effects of E2 on DC differentiation were hypothesized to be mediated by ER $\alpha$  because most of the known biological effects of E2 are mediated by this ER subtype (48). In contrast to our prediction, deletion of ER $\alpha$  in vivo did not significantly alter populations of CD11c<sup>+</sup> splenocytes in Esr1<sup>-/-</sup> females as compared with WT female mice. Although ER $\alpha$  may mediate many of the effects of E2 on DCs and IKDCs, if E2 concentrations are sufficiently high, then ER $\beta$  may compensate and mediate the effects of E2 on DC activity, as noted previously (14). In the present study, Esr1<sup>-/-</sup> females were not ovariectomized before experimentation. Therefore, although signaling through ER $\alpha$  was compromised, concentrations of E2 as well as signaling through ER $\beta$  were intact in Esr1<sup>-/-</sup> females. Future studies will delineate the precise roles of ER $\alpha$  and ER $\beta$  as well as the effects of ER $\alpha\beta$  heterodimers in mediating the effects of E2 on the functioning of DCs and IKDCs.

There currently is considerable interest in defining subpopulations of DCs, NK cells, and IKDCs based on surface marker profiles and cytokine activity (47, 51). Missing from current studies is an examination of the role that estrogens may play in the differentiation and activation of these closely related subpopulations of cells. In the present study, notable differences were observed between the in vitro effects of E2 on DC differentiation from BM precursor cells and the in vivo effects of E2 on the activity of mature CD11c<sup>+</sup> splenocytes. These data demonstrate that the precise effects of E2 on the phenotype and function of DCs depends on when during development these cells are exposed to E2. Exposure of BM precursors to E2 facilitates development of these cells into classic DCs that synthesize IL-12. Conversely, exposure of mature splenic DCs to sustained levels of E2 results in the expansion of an alternative phenotype of CD11c<sup>+</sup> cells that express CD49b and NK1.1 and enhance IFN- $\gamma$  production.

Notable differences were observed in vivo between ovx females treated with sustained concentrations of E2 and intact females with cyclical concentrations of E2, in which intact females had proportions of NK1.1<sup>low</sup> cells that were similar to ovx females. Whether there is a threshold concentration of E2 required to alter CD11c<sup>+</sup> populations requires investigation. Heightened production of IFN- $\gamma$  by CD11c<sup>+</sup> cells in response to E2 stimulation may facilitate innate and adaptive immune responses. Previous studies illustrate that CD11c<sup>+</sup>NK1.1<sup>+</sup> cells are a significant source of innate IFN- $\gamma$  and innate production of IFN- $\gamma$  by these cells is necessary for protection against *Listeria monocytogenes* (52). The observation that E2 enhances production of IFN- $\gamma$  by CD11c<sup>+</sup> cells provides a key mechanism regulating the downstream differences in adaptive immunity, development of autoimmune disease, and susceptibility to microbial pathogens between the sexes.

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