Estrogen Preferentially Promotes the Differentiation of CD11c+ CD11b intermediate Dendritic Cells from Bone Marrow Precursors

Vladislava Paharkova-Vatchkova, Ruben Maldonado and Susan Kovats

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Sex biases in autoimmunity and infection suggest that steroid sex hormones directly modulate immune cells. We show in this study that 17-β-estradiol (E2) promotes the differentiation of functional dendritic cells (DC) from murine bone marrow precursor cells. Remarkably, ex vivo DC differentiation was inhibited in steroid hormone-deficient medium, and was restored by addition of physiological amounts of E2, but not dihydrotestosterone. DC differentiation was inhibited by the estrogen receptor (ER) antagonists ICI 182,780 and tamoxifen, and from ERα−/− bone marrow cells, indicating that E2 acted via ERs. E2 addition was most effective in promoting DC differentiation immediately ex vivo, but did not increase DC proliferation. E2 treatment specifically promoted differentiation of a CD11c+ CD11bint DC population that displayed high levels of cell surface MHC class II and CD86, suggesting that E2 could augment numbers of potent APC. DC that differentiated in E2-supplemented medium were fully functional in their capability to mediate presentation of self and foreign Ags and stimulate the proliferation of naive CD4+ T cells. The requirement for estrogen during DC differentiation suggests a mechanism by which E2 levels in peripheral tissues might modulate both the number and functional capabilities of DC in vivo, thereby influencing immune responses. The Journal of Immunology, 2004, 172: 1426–1436.

Sex-based differences in immunity are suggested by gender differences in the incidence of autoimmune diseases (1). Lymphocyte-mediated autoimmune diseases in humans, e.g., multiple sclerosis, rheumatoid arthritis, Graves’ disease, systemic lupus erythematosus, and Hashimoto’s thyroiditis, and murine models of these diseases, such as experimental autoimmune encephalomyelitis and collagen-induced arthritis, are more prevalent in females than males. This sexual dimorphism also has been found in infectious disease pathogenesis (2–4). Sex hormone treatment of T cells or whole organisms has been shown to modulate autoimmunity and immune responses to pathogens in mouse models (5–9). Togethe with the finding that lymphocytes and myeloid cells express estrogen receptors (ER) and androgen receptors (AR) (6, 10), these observations suggest that the steroid sex hormones, estrogen and testosterone, could directly influence the function of cells involved in the immune response. Reports to date indicate diverse effects of sex hormones or their receptor antagonists on cellular differentiation, cytokine production, Th1/2 cell polarization, NO production, MHC class II (MHCII) expression, and APC recruitment or function (reviewed in Refs. 1 and 11). Although the underlying mechanism(s) by which sex hormones modulate immunity is not completely understood, estrogen levels currently are being manipulated during therapy for breast cancer and autoimmune disease (multiple sclerosis), and in postmenopausal women.

The 17-β-estradiol (E2) is a regulator of growth, differentiation, survival, or function in many target tissues, including the female and male reproductive systems, cardiovascular system, and bone (12). ER are ligand-dependent transcription factors that modulate gene transcription by binding coactivators or corepressors that vary in target tissues (13). In addition, rapid biological effects of E2 in some cell types suggest that E2 elicits nongenomic effects via cell surface ER linked to signal transduction pathways, e.g., mitogen-activated protein kinase (13). ERα and ERβ are members of the steroid/thyroid hormone superfamily of nuclear receptors; although both ER bind ligands with similar affinities, the conformationally distinct ligand/receptor complexes mediate different transcriptional effects (12). Both ERα and ERβ are expressed in the spleen and thymus (14). Disruption of the murine ERα or ERβ genes has revealed distinct roles for the two ER in reproductive, skeletal, and cardiovascular tissue (15). Endogenous ER ligands include estrone (E1), E2, and estriol (E3); E2 is the predominant form of estrogen present in males and nonpregnant females. The steroidal compound ICI 182,780 is a pure ER antagonist in all cell types (16). In contrast, selective ER modulators (SERM), such as tamoxifen, bind ER and differentially mediate ER-mediated transcription via the recruitment of cell type-specific coactivators or corepressors (17, 18).

Multiple studies have provided some evidence that APC numbers and functional capabilities are altered by normal or manipulated systemic levels of estrogen in vivo (19–24). Notably, numbers and function of both professional and nonprofessional APC in the female rat reproductive tract vary with different estrogen levels during estrus and diestrustr (25). A recent study showed that during development of experimental autoimmune encephalomyelitis, systemic E2 treatment suppressed disease symptoms and led to decreased numbers of dendritic cells (DC) migrating into the CNS at

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disease onset, and decreased DC production of TNF-α, IL-12, and IFN-γ (23). In vitro, high concentrations (5–10 μM) of the non-steroidal antiestrogens toremifene and tamoxifen inhibited the GM-CSF- and IL-4-mediated differentiation of human ER− CD14+ monocytes into CD1a+ CD14− DC (10). These antiestrogens acted independently of the ER, because neither E2 nor the ER antagonist ICI 182,780 had an effect in this cell system. Conversely, a separate study reported that E2 or testosterone addition to human monocyte cultures increased the number of monocyte-derived DC (26). A consistent pattern of estrogen effects on APC does not emerge from these diverse studies; it is possible that differences between in vitro and in vivo systems may be explained partially by differential effects of low vs high doses of E2, or effects of systemic E2 treatment on the levels of other steroid hormones, e.g., progesterone, known to influence immune cell function (27–29). Thus, we chose to investigate a potential role for E2 in DC biology using a well-defined ex vivo culture system for the development of murine DC.

DC are potent APC involved in the initiation of immune responses by naive T cells and contribute to polarization of Th1 or Th2 cell responses (30) and maintenance of peripheral self-tolerance (31, 32). In peripheral tissues, immature DC acquire Ags, and upon an inflammatory and/or pathogen stimulus, migrate to the lymph nodes, whereupon the now mature activated DC express high levels of surface MHCII and CD86 and are capable of activating naive T cells (31). DC arise from both myeloid and lymphoid progenitors present in bone marrow (BM) (32, 33).

To determine how sex hormones might modulate DC differentiation or Ag-presenting function, we used an ex vivo BM culture system in which GM-CSF promotes DC differentiation from myeloid precursors (34). These BM cultures typically yield two populations of CD11c+ DC that display either high or intermediate surface expression of CD11b. To define the effects of estrogen and testosterone, BM cells were cultured for 7 days in steroid hormone-deficient medium supplemented with defined amounts of E2 or dihydrotestosterone (DHT). Remarkably, CD11c+ CD11b+CD STR differentiation in steroid hormone-deficient medium was significantly inhibited, and could be restored by addition of physiological amounts of E2 to the cultures. A threshold amount of E2 acting via ERs was required during ex vivo DC differentiation from BM precursors isolated from either male or female mice. The CD11c+ CD11b+CD STR DC produced by these culture conditions displayed higher intrinsic levels of MHCII and costimulatory molecules than CD11c+ CD11b+CD STR DC, and were capable of presentation of self and foreign Ags by MHCII molecules as well as stimulation of naive CD4+ T cell proliferation.

Materials and Methods

Cell culture medium and reagents

Regular culture medium (regular FCS (RegFCS)) was RPMI 1640 supplemented with 10% FBS (Omega Scientific, Tarzana, CA), 2 mM glutamine, 100 U penicillin/0.1 mg streptomycin/ml, 10 mM HEPES buffer, 50 μM 2-ME, and 1 mM sodium pyruvate. Hormone-deficient medium (charcoal-dextran-treated FCS (CDFCS)) was RPMI 1640 lacking phenol red, supplemented with 10% charcoal-dextran-treated FBS (Omega Scientific). The DC-GM serum-free medium (Stemgenix, Amherst, NY) was supplemented with 50 μM 2-ME. Stock solutions (0.01 M) in ethanol of E2 and DHT (both from Sigma-Aldrich, St. Louis, MO) were diluted into BM cultures at varying concentrations on days indicated in the figures. DHT was used because it is not a substrate for aromatase conversion to estrogen. Stock solutions of ICI 182,780 (1 mM) (Faslodex; AstraZeneca, Cheshire, U.K.) and tamoxifen (10 mM) (Sigma-Aldrich) in DMSO were prepared and diluted into BM cultures at varying concentrations in the presence or absence of exogenous E2. Addition of ethanol or DMSO alone to BM cultures did not change DC differentiation or surface marker expression relative to completely untreated cultures.

Generation of DC from BM cells

The 129S6, C57BL/6, and ERα−/− (B6.129-Estra1tm1Nio) mice (Taconic Farms, Germantown, NY) were housed at the City of Hope Animal Resource Center in accordance with institutional and federal guidelines. BM cells were isolated from the femurs and tibiae of 8- to 12-wk-old female and male mice, and cultured with GM-CSF according to published protocols (35). GM-CSF containing conditioned medium from J558L cells transfected with the murine GM-CSF gene (36) was used; the J558L cells were kindly provided by R. Steinman (The Rockefeller University, New York, NY). Briefly, on day 0, 2 × 10^6 total BM cells (including erythrocytes) were put in 10 ml medium containing GM-CSF (3.3% J558L conditioned medium v/v) in 100 mm bacteriologically petri dishes. On day 3, 10 ml of medium containing GM-CSF was added to the plates. On day 6, half of the culture supernatant was removed, and cells were spun and resuspended in a fresh medium containing GM-CSF. In some of the cultures, LPS (2 μg/ml) (from Escherichia coli serotype 055:B5) (Sigma-Aldrich) was added from day 6–7 or day 8–9 to mature the DC. Immature and mature DC were harvested on day 7–9. E2, DHT, or ER antagonists were added on days 0, 3, and 6, or as indicated in the figures.

mAbs and flow cytometry

Fluorochrome- or biotin-labeled mAbs specific for CD11c, CD11b, CD86, CD80, Gr-1, B220, CD4, and CD8α were obtained from BD PharMingen (San Diego, CA). PE anti-DEC205 was obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). mAbs specific for MHCII (Y3P) and macrophages (F4/80) were purified from hybridoma (American Type Culture Collection, Manassas, VA) supernatant and biotinylated. Purified and labeled mAbs specific for I-Aα/Es1–26 complexes (YAE) or I-Aα/invariant chain degradation intermediate (CLIP) complexes (15G4) were kindly provided by A. Rudensky (University of Washington, Seattle, WA). Biotinylated mAbs were detected with streptavidin-CyChrome or streptavidin-APC (BD Pharmingen).Appropriate fluorochrome or biotinylated isotype control mAbs of each species were used as negative controls. Cells were preincubated with mAb 2.4G2 (anti-CD16/32) to block Fc receptors, and labeled with mAbs in PBS, 5% heat-inactivated newborn calf serum, and 0.1% sodium azide. Labeled cells were run on a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software. To detect I-Aα/Es1–26 complexes, DC were incubated with 30 μM Es1–26–58 peptide for 3 h at 37°C before labeling with mAb YAE and Cy5-labeled donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cell sorting was performed on a Mo-Fluor cytometer (Cytomation, Fort Collins, CO) in the City of Hope National Medical Center Analytical Cytometry core facility.

T cell hybrid assays

T cell hybrids used to assess Ag presentation were specific for epitopes derived from exogenous Ags, IgM 377–392 (77.1) (37) and hen egg lysozyme (HEL) 74–88 (B04) (38), or the endogenous Ag rab5 86–101 (2.3) (37). Immature DC1+ DC from day 7 cultures (unfractionated or purified using anti-CD11c microbeads and the Miltenyi Biotec (Auburn, CA) magnetic separation column system) were incubated for 20 h with the native Ags IgM (2–5 μg/ml) or HEL (100–400 μg/ml). Subsequently, the cells were matured for 24 h with LPS, washed, and titrated (between 5 × 10^4 and 3 × 10^5/well) in wells before addition of T cells (10^5/well). No T cell responses were detected in the absence of Ag. For rab5-specific responses, T cells were incubated with variable numbers of DC for 20 h. For all three T cell hybrids, antigenic peptides were added to non-Ag-pulsed DC as a positive control for T cell reactivity. IL-2 production by the T cell hybrids was assessed by proliferation of HT-2 cells (39) using an Alamar blue colorimetric assay; results are expressed as arbitrary units of OD at 570 vs 600 nm, average of duplicate wells.

Allogeneic T cell assays

Immature CD11c+ DC from day 7 cultures of 129S6 BM cells, containing either RegFCS or CDFCS without or with supplemented E2, were used as an unfractionated population, or purified using anti-CD11c microbeads. DC were activated by incubation for 20 h with LPS and y irradiated (3000 rad), or were incubated with LPS for 3 h and parafomaldehyde fixed. Prepared APC were titrated in wells and incubated with 2–2.5 × 10^6 per well naive allogeneic CD4+ T cells that were isolated from a B10.M (H-2b) mouse by cell sorting. Proliferation of T cells was assessed by addition of 0.5 μCi/well of [3H]TdR during the last 16 h of an 86-h culture period. Stimulation index was calculated as: (cpm responder + stimulator)/cpm responder alone.
Labeling of cells with CFSE

Upon isolation, BM cells (2 × 10^7/ml) were labeled with 10 μM CFSE (Molecular Probes, Eugene, OR) from a 10 mM stock solution (in DMSO) for 10 min at 37°C. Cells were washed and put into culture, and intensity of CFSE signal was monitored every 2 days by flow cytometry. CFSE-labeled cells were labeled with biotin anti-CD11c and streptavidin-APC, as described above.

Detection of sex hormone receptors by immunoblotting

Cells from day 7 BM cultures or all cells from BM directly ex vivo were solubilized in Nonidet P-40 lysis buffer, and variable amounts (10–60 μg) of cell lysate/lane were electrophoresed on 8% SDS-polyacrylamide gels and transferred to nitrocellulose, as described (40). To detect sex hormone receptors, the nitrocellulose was probed with polyclonal anti-ER (MC-20), anti-ERβ (H-150), or anti-AR (N-20) from Santa Cruz Biotechnology (Santa Cruz, CA), and HRP-labeled goat anti-rabbit IgG (Jackson Immunoresearch Laboratories). Breast (MCF-7) and prostate (LNCaP) cancer cell lines known to express ER and AR were used as controls, and were kindly provided by S. Chen (City of Hope National Medical Center). Blots were developed using an ECL substrate (Amersham Biosciences, Piscataway, NJ) and exposure to film.

Results

Estradiol promotes DC differentiation from BM precursors

To determine an effect of E2 on DC differentiation or function, we used a well-defined ex vivo culture system for GM-CSF-mediated generation of DC from BM precursors (34). Unfractionated BM cells from 129S6 mice were incubated in GM-CSF, as described (35), and on day 7, cells were assessed for the surface molecules characteristic of murine DC by flow cytometry (Fig. 1A). Approximately 40–60% of cells in these cultures were CD11b^+ CD11c^+ DC that were F4/80^− Gr-1^− CD4^− CD8^− B220^− DEC-205^−, as is typical for some splenic DC (41). CD11c^+ DC could be subdivided based on levels of CD11b surface expression, CD11b^high and CD11b^int. The DC displayed the low levels of surface MHCII and

![Image of figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Estradiol promotes DC differentiation from BM precursors. A, E2 restores DC differentiation in CDFCS. Female 129S6 BM was cultured in RegFCS (RPMI, 10% FCS) or CDFCS (phenol red-free RPMI, 10% charcoal-dextran-treated FCS) ± 1 nM E2 for 7 days before assessment of CD11b and CD11c expression by flow cytometry. Shown are dot plots of anti-CD11b PE vs anti-CD11c FITC (left panels) and the corresponding forward light scatter vs side scatter plots (right panels). The percentages of CD11b^high or CD11b^int CD11c^+ DC are indicated. Isotype control mAbs (hamster IgG FITC or rat IgG PE) did not bind to any cells in the BM cultures (data not shown). Data are representative of nine independent experiments. B, Amounts of E2 normally present in FCS restore DC differentiation in CDFCS. BM was cultured in CDFCS with variable amounts of E2 for 7 days. Numbers of CD11b^high or CD11b^int CD11c^+ DC that differentiated at each E2 are shown from two representative experiments. C, DHT does not restore DC differentiation in CDFCS. BM cells were incubated in CDFCS ± DHT (100 nM) before determination of CD11c and CD11b expression. Results are representative of two independent experiments. D, The inhibition of DC differentiation in serum-free medium is restored by addition of E2. BM was cultured in DC-GEM serum-free medium ± variable doses (0.01–0.5 nM) of E2 for 7 days, followed by assessment of CD11c and CD11b expression. Results are representative of two independent experiments. E, DC in E2-supplemented CDFCS display typical DC surface markers. Surface expression of MHCIi or CD86 on resting (top panels) or LPS-activated (bottom panels) CD11c^+ DC present in cultures (shown in A) containing RegFCS (shaded histograms), CDFCS (thin lines), or E2-supplemented CDFCS (thick lines) was assessed by flow cytometry.
CD11c characteristic of immature, resting DC, and activation by bacterial LPS (2 µg/ml) for 18 h to affect DC maturation resulted in increased levels of surface MHCII and CD86 (Fig. 1E). The CD11c− (CD11bhigh or CD11bint) cells in the culture displayed the myeloid differentiation marker Gr-1, but not MHCII nor CD86 either before or after LPS activation, suggesting that they are DC precursors (data not shown).

We reasoned that the influence of E2 on DC differentiation would be determined most accurately if cells were cultured in steroid hormone-deficient medium that is composed of CDFCS in phenol red-free RPMI. Passage of RegFCS over charcoal-dextran reduces the level of steroid hormones (including E2, estrone, and testosterone) (42). Levels of E2 in RegFCS are typically ~8–11 pg/ml (0.03–0.05 nM), and charcoal-dextran treatment reduces E2 to levels below the sensitivity of standard E2 RIA (<5 pg/ml). Phenol red has weak estrogenic activity at 15–45 µM, the concentration at which it is present in RPMI medium; the $K_a$ of phenol red for the ER has been estimated to be $2 \times 10^{-7}$ M, 0.001% of the affinity of E2 (43).

In contrast to BM cells cultured in RegFCS, steroid hormone-deficient medium (CDFCS) does not support efficient DC differentiation, as indicated by the significant reduction of CD11b+ CD11c− immature DC on day 7; the majority of cells remain CD11b+ (Fig. 1A). Remarkably, addition of 0.01–1 nM E2 to cells in hormone-deficient medium on days 0, 3, and 6 of the BM culture, during medium and GM-CSF renewal, led to the recovery of CD11c− DC on day 7 (Fig. 1, A and B). The paucity of DC in CDFCS may be visualized on forward light scatter vs side scatter plots, in which the majority of cells present in the absence of E2 have lower forward and side scatter than is typical for DC (Fig. 1A). The number of differentiated CD11c+ DC was markedly augmented by addition of amounts of E2 (0.01–0.1 nM) comparable to that found in RegFCS (0.03–0.05 nM) (Fig. 1B). The CD11c+ CD11bint DC population was most significantly affected by E2 levels in the culture medium; this population was most clearly reduced in CDFCS and increased ~5-fold upon addition of E2 to CDFCS (Fig. 1B). The CD11c+ CD11bhigh population was less dependent upon E2, although an E2-mediated increase in numbers of this population was observed in some experiments; an example

**FIGURE 2.** E2 preferentially promotes the differentiation of CD11c+ CD11bint DC. A, Excess E2 doubles the percentage of CD11bint DC that develops in GM-CSF-treated BM cultures in RegFCS. Female 129S6 BM cells were incubated ± 10 nM E2 for 7 days before assessment of CD11b and CD11c surface expression. The percentage of cells in each boxed region is indicated. Results are representative of 13 independent experiments. B, The number of differentiated CD11c+ DC was markedly augmented by addition of amounts of E2 (0.01–0.1 nM) comparable to that found in RegFCS (0.03–0.05 nM) (Fig. 1B). The CD11c+ CD11bint DC population was most significantly affected by E2 levels in the culture medium; this population was most clearly reduced in CDFCS and increased ~5-fold upon addition of E2 to CDFCS (Fig. 1B). The CD11c+ CD11bhigh population was less dependent upon E2, although an E2-mediated increase in numbers of this population was observed in some experiments; an example
of each result is shown in Fig. 1B. Addition of 1 nM E2 only on day 6–7 during the period of LPS activation did not restore DC in the cultures (data not shown). Similar E2 dose responses were observed with both female and male BM cells. Addition of DHT or testosterone (≤100 nM) from day 0 of BM culture did not restore the differentiation of DC (Fig. 1C and our unpublished observations).

Because the low amount of E2 remaining in CDFCS cannot be measured accurately using typical E2 RIA (HyClone Laboratories, unpublished observations), we assessed GM-CSF-induced DC differentiation in a defined serum-free medium (DC-GM) that lacks steroid hormones. Very few DC developed in DC-GM, and the viability of the cultures was poor. Addition of 0.01–0.1 nM E2 increased the number of differentiated DC ∼5-fold, indicating that amounts of E2 normally present in FCS (0.03–0.05 nM) are sufficient to promote DC differentiation (Fig. 1D). Amounts of E2 above 0.1 nM did not further augment DC recovery. However, viability of the cultures with added E2 remained poor, and the total number of cells recovered was less than numbers recovered in CDFCS cultures, suggesting that serum-free medium lacks other factors needed for viability of all cell types in BM cultures.

The DC in the E2-supplemented CDFCS displayed higher surface CD86 expression than observed on DC differentiated in regular medium, while expression of MHCII and CD86 after LPS activation was similar in both cultures (Fig. 1E). The small numbers of CD11b<sup>+</sup> CD11c<sup>`</sup> cells that did develop in CDFCS without added E2 (Fig. 1A) appear to be DC because they also expressed MHCII and CD86 (Fig. 1E); these cells may develop in response to the low amounts of E2 remaining in the hormone-deficient medium, or their differentiation may be E2 independent. These data obtained from culture of BM in CDFCS or serum-free medium indicate that E2 promotes DC differentiation by acting on DC precursors present in freshly isolated BM, and suggest that a threshold amount of E2 is required to effect differentiation.

**E2 preferentially promotes the differentiation of CD11c<sup>+</sup> CD11b<sup>`</sup> DC**

The DC-promoting effect of E2 also was observed in RegFCS. The presence of E2 (0.01–10 nM) significantly increased the number of differentiated CD11c<sup>+</sup> CD11b<sup>`</sup> DC, concomitant with a reduction of CD11b<sup>+</sup> CD11c<sup>`</sup> cells (Fig. 2, A and B). In 13 independent experiments in which BM cultures in RegFCS from individual male or female mice were supplemented with 10 nM E2, a 10–50% increase in numbers of CD11c<sup>`</sup> DC was observed (geometric mean ratio E2/control 1.26). E2 markedly induced the accumulation of CD11b<sup>`</sup> DC, while the number of CD11b<sup>`</sup> DC was only slightly increased by E2 addition. Compared with CD11b<sup>`</sup> DC, CD11b<sup>`</sup> DC display higher cell surface MHCII and CD86 before and after LPS activation (Fig. 2C). In addition, differentiation in excess E2 augmented the MHCII and CD86 on immature CD11b<sup>`</sup> and CD11b<sup>`</sup> DC (Fig. 2C).

To determine whether the presence of E2 was required during the entire 7-day culture period to promote DC differentiation, E2 was added to BM cells on different days after isolation (day 0, 1, 3, or 5) and retained in the culture from the day of addition until the cells were harvested on day 7. Exogenous E2 was most effective in augmenting CD11b<sup>`</sup> DC differentiation when added from day 0 of culture; the differentiation-promoting effects of E2 decreased daily as the culture progressed; numbers of CD11b<sup>`</sup> cells remained relatively constant (Fig. 2D). Thus, it is likely that E2 acts on cells present early in the culture period; as the culture progresses, such precursor cells appear to be depleted or have insufficient time to complete differentiation.

To confirm that the two distinct CD11c<sup>+</sup> populations distinguishable by differential CD11b expression in these BM cultures are DC, DIC microscopy was used to assess cell morphology. CD11c<sup>+</sup> CD11b<sup>`</sup> and CD11c<sup>`</sup> CD11b<sup>`</sup> populations were sorted with a flow cytometer and plated in dishes for several hours before microscopy. Cells from both CD11c<sup>+</sup> CD11b<sup>`</sup> and CD11c<sup>`</sup> CD11b<sup>`</sup> populations that were present in BM cultures containing RegFCS or CDFCS + E2 (0.1 nM) exhibit the dendrites characteristic of DC (Fig. 3). These morphological data corroborate our cell surface marker and functional data (see below) to indicate that the cells that differentiate in E2-supplemented CDFCS are DC.

**BM-derived DC express ER, but not AR**

E2 was effective at promoting DC differentiation in CDFCS at 0.1–1 nM (27–272 pg/ml), at the K<sub>E</sub> of the ER, consistent with the concentration of E2 in adult mouse serum during female diestrus.

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**FIGURE 3.** CD11b<sup>`</sup> and CD11b<sup>`</sup> DC exhibit characteristic dendritic morphology. DC populations were sorted from day 7 BM cultures, and DIC images of live cells were obtained with an Olympus AX70 microscope, original magnification ×400. Shown are A, CD11c<sup>+</sup> CD11b<sup>`</sup> DC or B, CD11c<sup>`</sup> CD11b<sup>`</sup> DC from BM cultures containing CDFCS + 0.1 nM E2. Shown are C, CD11c<sup>+</sup> CD11b<sup>`</sup> DC or D, CD11c<sup>`</sup> CD11b<sup>`</sup> DC from BM cultures containing RegFCS.
FIGURE 4. BM cells and DC express ERα and ERβ. A–C, Immunoblots containing variable amounts (10–60 μg) of cell lysate isolated from all cells in DC cultures on day 7 were probed with rabbit antisera specific for ERα, ERβ, or AR, as indicated. For ER α blots, control lysate was from the breast cancer cell line MCF-7 that expresses ERα isoforms ranging between 63 and 78 kDa and ERβ isoforms ranging between 56 and 62 kDa; for the AR blot, control lysate was from the prostate cancer cell line LnCaP, which expresses the 110-kDa AR. D, Female (BM-F) or male (BM-M) BM cells were lyzed immediately upon isolation, and 20 μg of cell lysate was electrophoresed, transferred to nitrocellulose, and probed with a rabbit antiserum specific for ERα.

(25–35 pg/ml) and estrus (100–200 pg/ml) and in males (8–15 pg/ml) (15, 44). Conversely, DHT did not promote DC differentiation at doses between 10 and 1000 nM. The Kd of the AR also is 0.1–1 nM (29–290 pg/ml), and normal serum levels of testosterone are 0.1–0.8 ng/ml in females and 5–15 ng/ml in males (15, 44). Female and male cells in GM-CSF-supplemented BM cultures on day 7 were shown to contain known ER, ERα, and ERβ, but not the AR, by immunoblotting with specific antisera (Fig. 4, A–C). Unfractionated BM cells analyzed directly ex vivo also expressed ER (Fig. 4D).

ER antagonists inhibit DC differentiation

To demonstrate that E2 is acting via the ER to promote DC differentiation in CDFCS, the steroidal ER antagonist ICI 182,780 was titrated into cultures containing 1 nM E2 (Fig. 5A). As the amount of ICI 182,780 increased between 1 and 100 nM, the number of DC developing in BM cultures supplemented with both E2 and ICI 182,780 decreased 3- to 4-fold, indicating that E2 was competitively inhibited by ICI 182,780 and thus was acting via the ER to promote DC differentiation. The numbers of DC that differentiate in CDFCS alone also were reduced by addition of ICI 182,780, suggesting that this differentiation is E2 dependent.

To confirm that differentiating DC require the E2 present in RegFCS, titrated amounts (1–100 nM) of ICI 182,780 were added to BM cultures containing RegFCS (Fig. 5B). Treatment of BM cells from day 0 with ICI 182,780 results in a 3- to 4-fold reduction in the number of CD11bint DC on day 7. The CD11bint DC were not significantly affected by ICI 182,780. These data indicate that ICI 182,780 preferentially inhibits the differentiation of the CD11bint DC population and that these cells respond to E2 present in RegFCS via ER. Culture of BM cells with both ICI 182,780 (1–100 nM) and E2 (10 nM) showed that a 2- to 10-fold excess of ICI 182,780 was required to inhibit the DC-promoting effect of E2 (data not shown).

The SERM tamoxifen may act as an agonist or an antagonist depending on the cell type (17). Because long-term systemic treatment of tamoxifen is used for breast cancer prevention, it was of interest to determine the effects of tamoxifen on DC differentiation. The Kd of tamoxifen for the ER is ~6 nM, or 3% of E2 (43); plasma levels typically achieved in human patients are ~200 nM. When present during the 7-day culture period in medium containing RegFCS, tamoxifen (10–500 nM) reduced the number of differentiated DC, with a greater reduction of CD11bint cells at the lower doses (Fig. 5C). Tamoxifen competed with E2 for effect, indicating its action via the ER, and has antagonist activity in BM cells, consistent with our observation that tamoxifen does not restore DC differentiation in CDFCS (data not shown).

Treatment of cultures with ICI 182,780 or tamoxifen led to a decrease in MHCII and CD86 on CD11c+ CD11bint cells that remained in the cultures (Fig. 5D). Tamoxifen disrupts the pH of endocytic vesicles independently of the ER (45), which could inhibit intracellular trafficking of MHCII; however, reduction of MHCII and CD86 was similar in ICI 182,780 and tamoxifen-treated cultures, suggesting that it occurred as a consequence of ER...
DC differentiation from BM cells lacking ERα is inhibited

BM cells express both ERα and ERβ. To determine a role for ERα in DC differentiation, BM was isolated from female ERα−/− (B6.129-Estra-m1 N10) or control C57BL/6 mice and incubated without or with E2 (10 nM) in RegFCS (Fig. 6). DC differentiation from the ERα−/− BM (22% CD11c−) cells) was reduced significantly compared with C57BL/6 BM (52% CD11c− cells), with the greatest reduction in CD11bhi cells (Fig. 6, A and D). The majority of DC that did develop from the ERα−/− BM were CD11bint, a phenotype remarkably similar to control BM treated with ICI 182,780 (Fig. 6C). Addition of 10 nM E2 to the ERα−/− BM cells resulted in differentiation of near normal numbers of both DC populations, comparable to DC numbers derived from untreated C57BL/6 BM, but less than from E2-treated C57BL/6 BM (Fig. 6, B and E). Similar data were obtained upon analyses of multiple ERα−/− and C57BL/6 mice (2 females and 2 males of each strain). These data indicate that ERα has an important role in mediating E2 responsiveness in DC precursors; however, if the ERα−/− DC precursors are exposed to sufficient amounts of E2, then ERβ is capable of mediating those E2 responses, resulting in DC differentiation.

Estrogen promotes DC differentiation without increasing DC proliferation

Our data with E2-supplemented regular and hormone-deficient medium suggest that E2 promotes DC differentiation from precursor cells, or the survival of DC or DC precursors. Alternately, E2 could induce more rapid proliferation of already differentiated DC. To monitor proliferation in BM cultures, cytosolic proteins in BM cells were labeled on day 0 with the dye CFSE. BM cells incubated in the absence or presence of E2 were analyzed on days 1, 3, 5, and 7 for intensity of CFSE and CD11c expression by flow cytometry (Fig. 7, A–C). Although E2-treated cultures had more CD11c− cells by day 3, the intensity of the CFSE signal decreased in the two populations equally between days 1 and 7, indicating that cells were dividing in the culture at similar rates. These data suggest that E2 promotes DC differentiation or survival, rather than increasing proliferation of already existing DC.

Estradiol-treated DC retain the ability to degrade and present self and foreign Ags via MHCII

To determine whether E2 altered the function of DC, the intrinsic ability of DC to process and present Ags via MHCII was monitored using T cell hybrid as specific probes for peptide/MHC complexes. Because addition of E2 increases the number of DC, CD11c− DC were purified from BM cultures (≤10 nM E2) in RegFCS on day 7 using anti-CD11c magnetic beads. Purified DC from control and E2-treated cultures displayed equivalent levels of surface MHCII and CD86 after LPS activation; therefore, our T cell hybrid assay directly measured the presentation of specific peptide/MHCII complexes. DC were incubated with the exogenous Ags HEL and IgM, activated by LPS, titrated in wells, and incubated with T cell hybrids specific for HEL 74–88 and IgM 377–392 epitopes. No significant differences were observed in the ability of control or E2-treated DC to present these Ags, indicating that E2-treated DC are fully functional (Fig. 8, A and B). Presentation of an epitope derived from the endogenous self Ag rab5 also was identical in control and E2-treated DC (Fig. 8C). These data indicate that while E2 promotes the differentiation of DC, resulting in increased DC numbers and increased Ag-presenting function on a population level (data not shown), individual mature DC that had differentiated in the presence of E2 do not differ in intrinsic ability to present model Ags to T cells.

DC that differentiate in E2-supplemented CDFCS stimulate proliferation of allogeneic naive T cells

To determine whether the DC that had differentiated in E2-supplemented (0.1 nM) CDFCS were functional, we assessed the ability of purified, LPS-activated CD11c+ DC (129S6, H-2b) to stimulate proliferation of naive allogeneic (B10.M, H-2f) CD4+ T cells (Fig. 9). Compared with CD11c+ DC present in BM cultures containing RegFCS, CD11c+ DC from E2-supplemented CDFCS exhibited superior ability to induce the proliferation of naive CD4+ T cells (Fig. 9A). Because the CD11c+ CD11bint DC display higher levels of cell surface CD86, this observation may be explained in part by the greater number of CD11c+ CD11bint DC in the cultures containing E2-supplemented CDFCS (50% CD11bint) than in cultures containing RegFCS (38% CD11bint). The geometric means of CD86 expression on the combined CD11c+ DC populations were 144 in CDFCS and 71 in RegFCS. To determine whether the small numbers of DC that differentiated in CDFCS...
cubated with naive CD4+/H11001 not vary significantly on each day.

FIGURE 7. E2 promotes DC differentiation without increasing DC proliferation. A. Control and E2-treated DC divide at similar rates. BM cells were labeled with CFSE on day 0 and cultured ±E2 (10 nM) in RegFCS for 7 days. An aliquot of cells from each culture was taken on day 1, 3, 5, or 7 and analyzed for intensity of CFSE and CD11c expression. B. The percentages of CD11c+ DC on each day (±E2) are plotted; CD11c levels greater than fluorescence intensity of 25 (log scale) were considered positive for this graph. C. The total number of cells in each culture (±E2) did not vary significantly on each day.

FIGURE 8. Estradiol-treated DC function to present Ags via MHCII. LPS-activated DC differentiated in RegFCS in the presence of excess E2 retain the intrinsic ability to present Ag via MHCII. Sorted CD11c+ DC from control or E2-treated cultures, displaying comparable amounts of cell surface MHCII and CD86, were incubated overnight with A, soluble IgM (2 μg/ml) or B, HEL (400 μg/ml), followed by LPS activation, titration in wells, and incubation with Ag-specific T cell hybrids. C, LPS-activated DC also were incubated with T cells specific for the self Ag rab5. IL-2 production of T cells was assessed by HT-2 proliferation using an Alamar blue colorimetric assay; results are expressed as arbitrary units of OD at 570–600, average of duplicate wells. Responses of the T cells to APC in the absence of Ag were <0.01 OD570–600. Cognate peptide (1 μg/ml) was added to LPS-activated DC (400 cells) at the time of the T cell assay and resulted in >0.5 OD570–600. Results are representative of two independent experiments.

Discussion

Sex biases in lymphocyte-mediated autoimmune diseases and APC expression of ER and AR suggest that sex hormones could directly influence APC. To determine a role for sex hormones in DC differentiation and function, we provided E2 or DHT during ex vivo differentiation of DC from murine BM cells in steroid hormone-deficient medium. Remarkably, efficient DC differentiation was not supported by CDFCS or serum-free medium, and was restored upon addition of amounts of E2 normally found in FCS and mouse serum, while DHT did not have this effect. The CD11c+ cells in these BM cultures may be divided into two groups based on the magnitude of CD11b expression (CD11bhigh vs CD11binf); immature CD11binf DC exhibited higher, more uniform MHCII and CD86 expression than immature CD11bhigh DC. Addition of E2 induced selective accumulation of CD11binf DC, while CD11bhigh DC numbers were less affected by E2 levels. Conversely, treatment with ER antagonists or the absence of ERα primarily blocked the accumulation of the CD11bhigh DC. Thus, E2 preferentially promotes the development of one of two types of DC, distinguishable by levels of CD11b expression, that differentiate in GM-CSF-supplemented BM cultures.

Our data support the hypothesis that E2 promotes the differentiation of DC from precursors in the BM. During the 7-day culture period, as the number of CD11c+ CD11bhigh DC increases, CD11c− CD11bhigh cells are depleted. Recent work of others has shown that CD11c− cells present on day 7 of GM-CSF-supplemented BM cultures include direct precursors of CD11c+ cells (46). Monitoring of cell division using the cytosolic dye CFSE showed that E2 did not increase proliferation of differentiated CD11c+ DC, suggesting that E2 promotes the development of CD11c+ cells at the expense of CD11bhigh CD11c− precursor cells. Furthermore, addition of E2 has maximal effect at the beginning of the culture, consistent with E2 action on precursor cells rather than already differentiated DC. Alternately, E2 may regulate the survival of differentiated DC.

The influence of E2 on hematopoietic cell differentiation is not unprecedented, because sustained elevated systemic E2 (or DHT) selectively depletes lymphoid-restricted progenitors (Lin− IL7Rα− c-kitlow TdT+) from murine BM and the thymus (47, 48). Conversely, decreased levels of systemic sex steroids resulted in greater numbers of BM B cell precursors. Although B and T cell precursors were decreased in the Lin− c-kithigh fraction of BM of mice with elevated systemic E2, myeloid precursors were present in normal numbers, and a common myeloid progenitor population...
Figure 9. DC that differentiate in E2-supplemented CDFCS stimulate the proliferation of naive allogeneic CD4^+ T cells. A, CD11c^+ DC (12086, H-2^d) were purified from cultures containing CDFCS + 0.1 nM E2 or RegFCS (■). LPS activated to induce maturation and titrated in wells before addition of purified CD4^+ T cells (B10.M, H-2^h). Unfractionated immature DC from CDFCS (E2) cultures were used as a negative control (□). B, purified CD11c^+ DC from cultures containing CDFCS + 0.1 nM E2 or unfractionated cells (containing 15% CD11c^+ DC) from CDFCS (E2) cultures (○) were LPS activated and titrated in wells before addition of purified CD4^+ T cells. Unfractionated immature DC from CDFCS (+E2) cultures were used as a negative control (triangles). Proliferation of T cells was assessed by addition of 0.5 μCi/well of ^3HTrd during the last 16 h of an 86-h incubation period. Proliferation of T cells alone or APC alone was <100 cpm. Numbers next to symbols are the stimulation index at the indicated responder to stimulator ratio.

(Lin^- c-kit^high Sca-1^- IL-7Rα^- CD34^+/--/ FcRIII/III^+/-- ) was unchanged. Lin^- c-kit^high cells with potential for lymphoid or myeloid differentiation were found to be directly sensitive to E2 in serum- and stromal-cell-free in vitro cultures supplemented with stem cell factor, IL-7, and Flt2/Flik3 ligand (49). E2 addition (10 nM) to both Lin^- c-kit^high and c-kit^lowm musors resulted in inhibition of CD19^+ B cells, but an increase (up to 2.5-fold) in Gr-1^- myeloid cells, and elevation of absolute numbers of CD45R^-CD19^- cells. Taken together with our data, these observations suggest that E2 has opposing effects on BM lymphoid and myeloid differentiation, and that E2 may promote DC differentiation from a Lin^- c-kit^high myeloid progenitor (50).

E2 acted via the ER on DC precursors because the ER antagonists ICI 182,780 and tamoxifen blocked the differentiation of the E2-responsive DC population and competed with E2 for effect. CD11c^+ CD11b^int DC differentiation from BM cells isolated from ERα^/- mice also was inhibited, but could be restored partially by exogenous E2. Thus, our data with BM cultures from ERα^/- mice suggest roles for both ERα and ERβ in DC differentiation, although ERβ is unable to efficiently compensate for the lack of ERα unless the cultures are supplemented with additional E2. These data support a model in which E2-regulated genes required for DC differentiation are more efficiently activated by ERα than ERβ; thus, exposure to higher levels of E2 is required for ERβ-mediated gene expression. Alternately, the E2-responsive myeloid precursors may have lower numbers of ERβ than ERα; relative numbers of the two ER vary among cell types (14). ERα was shown previously to have a role in murine hemopoietic stem cell development and B cell maturation (51).

Other steroid hormones have been shown to modulate human monocyte to DC differentiation or murine DC differentiation from BM in vitro. Although glucocorticoids and vitamin D3 inhibit DC differentiation (52–54), the thyroid hormones triiodothyronine and thyroxine were shown to promote DC differentiation from monocytes via an unknown mechanism (55). However, we did not observe an effect of these thyroid hormones in the murine BM DC culture system (R.M., unpublished data). Future dissection of signal transduction and gene regulatory pathways downstream of the ER and other steroid hormone receptors in DC precursors will lead to a greater understanding of molecular events underlying DC differentiation.

The relationship between the two DC populations observed in these GM-CSF-supplemented BM cultures remains unclear; however, our data suggest that the CD11c^+ CD11b^int population contains the more potent APC. Immature CD11b^int DC exhibited higher, more uniform MHCI and CD86 expression than CD11b^high DC, and showed a greater increase in cell surface MHCI and CD86 after activation with bacterial LPS. E2 supplementation of BM cultures increased the overall levels of MHCI and CD86 on CD11c^+ DC in two ways: 1) by increasing the number of CD11b^int DC, and 2) by increasing the level of cell surface MHCI and CD86 on immature CD11b^high and CD11b^int DC. A similar increase in CD86 was found on immature DC that developed in E2-supplemented CDFCS. In contrast to effects on MHCI and CD86, E2 did not induce expression of CD40 on immature DC, nor change CD40 expression on LPS-activated DC (unpublished data). Addition of E2 to cultures only on day 6–7 during LPS activation did not increase surface expression of MHCI nor CD86, suggesting E2 does not directly modulate signals resulting from LPS ligation of Toll-like receptors that ultimately lead to CD86 up-regulation (unpublished data). The combination of 10 nM E2 and very low amounts of LPS (0.2 ng/ml) did not increase CD86 nor MHCI (data not shown), indicating that E2 does not synergize with suboptimal amounts of LPS to affect DC maturation.

Mechanisms that account for the increased MHCI expression on LPS-activated DC may apply to immature DC that develop in E2-supplemented cultures: increased MHCI synthesis, invariant chain degradation, peptide loading, or transit of MHCI molecules from intracellular vesicles to the cell surface (31). For example, inflammatory stimuli normally lead to fewer MHCI molecules bound to the invariant chain degradation intermediate CLIP and greater numbers of MHCI molecules bound to high affinity self or foreign peptides (31). To determine whether E2 affects the self peptide repertoire of surface MHCI molecules, DC were assessed for binding of the anti-CLIP/I-A^b mAb 15G4 (56). Although E2-treated immature DC display higher surface MHCI than control DC, the amount of mAb 15G4 staining was identical on the two DC populations (unpublished data), suggesting that E2-treated DC contained a lower portion of MHCI molecules bound to CLIP. To determine whether surface MHCI on E2-treated DC contained more tightly bound high affinity peptides, the ability of surface MHCI to bind exogenously supplied peptides was monitored. DC incubated with the Ec52–68 peptide showed no difference in binding of the Ec52–68/I-A^b complex-specific mAb YAe (57); thus, a dramatic change in the affinity of surface MHCI-bound peptides did not result from E2 treatment (our unpublished data).

Our experiments show that E2 promotes DC differentiation, resulting in increased numbers of CD11c^+ CD11b^int DC that, upon LPS activation, display higher levels of MHCI and CD86 than the CD11c^+ CD11b^high DC population. In vivo, the E2 produced in extragonadal sites, e.g., bone or adipose tissue, is thought to exert its biological influence locally (58), and therefore the local concentrations of E2 in peripheral tissues may increase numbers of CD11c^+ CD11b^int DC that display augmented levels of MHCI and costimulatory molecules, leading to their greater presentation of self Ags. Our data suggest a mechanism by which E2 could enhance overall DC Ag-presenting function in vivo in the absence of inflammation and thus promote autoimmunity. E2 may render immature tissue DC more likely to achieve expression levels of MHCI/peptide complexes and costimulatory molecules necessary to overcome naive T cell self-tolerance. Interestingly, in a murine model of SLE, systemic E2 elevation alters thresholds for B cell...
apoptosis and activation, resulting in accumulation of autoreactive B cells that would be deleted in normal mice (59). Conversely, decreases in systemic E2 due to SERM treatment of hormone-responsive breast cancer may lead to reduced DC numbers in these patients (60). In view of our data, therapeutic antitumors may decrease numbers of DC capable of Ag presentation, thereby limiting both pathogen and tumor immunity. SERM bind ER and differentially modulate ER-mediated transcription via the recruitment of cell type-specific coactivators or corepressors (13, 18). Thus, an ideal SERM for cancer prevention would have agonist activity in bone and myeloid cells, but antagonist activity in reproductive tissues.

Our observations will lead to a greater understanding of the molecular requirements for differentiation of DC, as well as how estrogen might influence immune responses in vivo. Sex hormones currently are being manipulated during therapy for sex hormone-dependent cancers and autoimmune diseases such as multiple sclerosis, and in postmenopausal women (60, 61). Such hormone therapies may alter DC differentiation and function, leading to either desired or detrimental effects on DC presentation of tumor Ags or autoantigens.

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