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The Selective Estrogen Receptor Modulators, Tamoxifen and Raloxifene, Impair Dendritic Cell Differentiation and Activation

Greg Nalbandian, Vladislava Paharkova-Vatchkova, Allen Mao, Stephanie Nale, and Susan Kovats

Most immune cells, including myeloid progenitors and terminally differentiated dendritic cells (DC), express estrogen receptors (ER) making these cells sensitive to estrogens. Our laboratory recently demonstrated that 17-β-estradiol (E2) promotes the GM-CSF-mediated development of CD11c⁺CD11b⁺⁺⁺⁺⁺ DC from murine bone marrow precursors. We tested whether the therapeutic selective estrogen receptor modulators (SERM), raloxifene and tamoxifen, can perturb DC development and activation. SERM, used in treatment of breast cancer and osteoporosis, bind to ER and mediate tissue-specific agonistic or antagonistic effects. Raloxifene and tamoxifen inhibited the differentiation of estrogen-dependent DC from bone marrow precursors ex vivo in competition experiments with physiological levels of E2. DC differentiated in the presence of SERM were assessed for their capacity to internalize fluoresceinated Ags as well as respond to inflammatory stimuli by increasing surface expression of molecules important for APC function. Although SERM-exposed DC exhibited increased ability to internalize Ags, they were hyporesponsive to bacterial LPS: relative to control DC, they less efficiently up-regulated the expression of MHC class II, CD86, and to a lesser extent, CD80 and CD40. This phenotype indicates that these SERM act to maintain DC in an immature state by inhibiting DC responsiveness to inflammatory stimuli. Thus, raloxifene and tamoxifen impair E2-promoted DC differentiation and reduce the immunostimulatory capacity of DC. These observations suggest that SERM may depress immunity when given to healthy individuals for the prevention of osteoporosis and breast cancer and may interfere with immunotherapeutic strategies to improve antitumor immunity in breast cancer patients. The Journal of Immunology, 2005, 175: 2666–2675.

Dendritic cells (DC)⁴ are bone marrow (BM)-derived professional APC that control the development of immune responses through the activation and polarization of naïve T cells (1). DC acquire Ags when immature for presentation in the context of MHC proteins but are relatively nonimmunogenic APC in this state. To effectively stimulate naïve T cells, DC undergo maturation in the presence of inflammatory stimuli or pathogen products, a process characterized by marked up-regulation of cell surface peptide loaded MHC proteins and costimulatory molecules such as CD80, CD86, and CD40 (2). Furthermore, mature DC produce cytokines like IL-12, IFNs, and IL-10 that control inflammation and contribute to DC-mediated T cell polarization to either a Th1 or Th2 type (2, 3). In vivo maturation and cytokine secretion can be triggered when microbial components are bound by TLR and this in part allows for the connection between the innate and adaptive immune systems (4). Binding of a particular microbial product to its corresponding TLR affects Th polarization, which determines the type of immune response that is activated by DC (2). Thus, immunity is profoundly controlled by DC and therefore, indirectly by factors that affect the differentiation, function, and survival of these important cells. Hormones, as well as cytokines, are two such types of factors that regulate immune function through their effects on DC and other cells of the immune system (5–8).

Both lymphoid and myeloid cells express estrogen receptors (ER), and the steroid sex hormone estrogen has been recognized for its influence on immune cells as a growth and differentiation factor with effects on hemopoiesis, lymphocyte activation, Th polarization, and cytokine production (reviewed in Ref. 9). In general, many estrogen-mediated effects can be modulated by a class of synthetic compounds called selective estrogen receptor modulators (SERM). These ER-binding compounds are termed selective modulators because they can compete with estrogen, acting as either agonists or antagonists of ER function in a tissue-dependent manner (10–12). There are two estrogen receptors, ERα and ERβ, which act as ligand dependent transcription factors that once bound to ligand, translocate into the nucleus, and in conjunction with coactivators or corepressors, modulate gene expression (13). There also may be rapid, nongenomic signaling pathways that are activated by ER (13). Interestingly, ER-ligand complexes are reported to regulate the activity of NF-κB (14), an important transcription factor for many aspects of immune function including TLR signaling (15).

Currently two SERM, tamoxifen and raloxifene, are used by physicians for their clinical benefits (16, 17). Tamoxifen, which has been used as a preventative and therapeutic agent, blocks the growth of estrogen-dependent breast cancers but increases the risk.
of endometrial cancer. Thus, it has opposing effects on estrogen dependent growth; it operates like an ER antagonist in the breast yet as an ER agonist in the endometrium. Raloxifene, like estrogen, supports the maintenance of bone density and is used for the treatment of osteoporosis. In addition to this agonistic effect in bone, clinical trials showed that raloxifene treatment decreased the incidence of both breast and endometrial cancer, suggesting that it acts as an ER antagonist in the breast and the endometrium. Thus raloxifene may be an attractive alternative to tamoxifen for the treatment of breast cancer, and the ongoing Study of Tamoxifen and Raloxifene (STAR) trial is directly comparing the efficacy of these two SERM for breast cancer prevention. Despite the widespread clinical application of tamoxifen and raloxifene, very little is understood about how they might affect the function of ER-expressing immune cells.

Several studies indicate that SERM influence multiple aspects of the immune system. SERM have been shown to modulate a number of pro- and anti-inflammatory cytokines (18–21). Furthermore, in vivo exposure of mice to tamoxifen (22) or raloxifene (23) reduced lymphoid organ weights suggesting that these SERM may dampen immune responses. In agreement with this idea, raloxifene negatively regulated B lymphopoiesis in BM in two separate studies (24, 25), and tamoxifen treatment was shown to reduce the severity of autoimmune disease in mouse models (18, 22, 26). However, the observation that tamoxifen has beneficial effects on autoimmune disease are contradicted by data suggesting that tamoxifen can augment lymphocyte activation (22, 27). Regardless, because ER are expressed by many cells of the immune system, including DC (28–31), it should not be surprising that SERM possess immunomodulatory properties.

There is now a growing body of evidence to suggest that estrogens can directly affect the development and performance of APC (reviewed in Ref. 9) supporting our hypothesis that SERM also may affect DC differentiation or function. Two studies have shown that the SERM, tamoxifen and toremifene, between 5 μM and 5 mM inhibited the GM-CSF- and IL-4-supported differentiation of human DC from peripheral blood monocytes and synovial fluid macrophages (28, 32). Differentiation of monocytes in the presence of these two SERM resulted in fewer cells expressing the DC marker, CD1a, and the DC that did develop exhibited impaired maturation in response to LPS and TNF-α as evidenced by significant inhibition of HLA-DR and CD83 up-regulation. The DC also were impaired in their ability to produce biologically active IL-12 following CD40 ligation and to stimulate the proliferation of allogeneic PBMC. Based on competition experiments with 17- β-estradiol (E2), it was concluded that SERM were not operating through ER because E2 was not found to counteract the inhibitory effects of SERM on DC phenotype. Our own studies have shown that the development of murine bone marrow-derived DC (BMDC) from GM-CSF-stimulated precursors in vitro was promoted by the presence of E2 in the culture medium (31). Differentiation of BMDC from E2-supplemented cultures was inhibited in the presence of a molar excess of the full ER antagonist, ICI 182,780, and tamoxifen. In contrast to the aforementioned studies, our data indicated that tamoxifen acted via ER to inhibit DC differentiation. The reasons underlying this disparity are unclear but might be related to differential requirements for E2 of human monocytes and murine BMDC progenitors.

These studies illustrate the potential for SERM to affect immune responsiveness through modulation of DC yet there have been no reports on the effects of raloxifene in this regard. To determine whether raloxifene acted like tamoxifen to inhibit DC differentiation and function, we compared their effects on DC generated in vitro from murine bone marrow progenitors in the presence or absence of E2. We report that like tamoxifen, raloxifene does not act as an ER agonist in BM progenitors, rather it acts as an antagonist to inhibit BMDC differentiation that is dependent upon the presence of E2. Those SERM exposed DC that did differentiate exhibited increased capacity to internalize Ags and were hyporesponsive to LPS activation as measured by decreased up-regulation of MHC class II and costimulatory molecules, suggesting that SERM acted to maintain DC in the immature state. These data suggest that raloxifene and tamoxifen may decrease the strength of immune responses in vivo through modulation of DC differentiation and activation.

Materials and Methods

DC culture reagents

DC were cultured in either hormone-deficient (DEF) or normal (NORM) RPMI 1640 medium. DEF medium contained 10% charcoal dextran-stripped FBS (Omega Scientific) lacking phenol red. Charcoal dextran stripping of FBS extracts steroid hormones and reduces their levels below the detection limits of a standard RIA (33). Phenol red was omitted because it can have weak estrogenic activity at the concentration that is present in RPMI 1640 medium (34). NORM medium contained 10% regular FBS (Omega Scientific) with phenol red. Aside from these two differences, both medium formulations were the same and consisted of 2 mM glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin. Medium conditioned by the GM-CSF-producing cell line, J558L (35), was added to DC culture medium at 5% to promote DC development. J558L cells were kindly provided by Dr. R. Steinman (Rockefeller University, New York, NY). Estrogen, in the form of E2 (Sigma-Aldrich), was solubilized in 95% ethanol and was diluted into DEF medium cultures at the concentrations indicated from a 1 μM stock solution. Stock solutions of tamoxifen, 4-hydroxytamoxifen (4OH), and raloxifene or comparable amounts of vehicle alone were prepared and added to cultures at the indicated concentrations. Tamoxifen (Sigma-Aldrich) stock solutions were prepared in DMSO or 95% ethanol at 10 mM; 4OH (Sigma-Aldrich) stock was prepared at a concentration of 50 mM in 95% ethanol, and raloxifene (Sigma-Aldrich) stock was prepared at a concentration of 10 mM in DMSO.

DC generation from murine BM

DC were generated from murine BM precursors as previously described (36). BM was isolated from 6- to 12-week-old male and female 129S6 mice (Tacionic), which were housed at the City of Hope Animal Resource Center (Duarte, CA) in compliance with federal and institutional guidelines. BM was flushed from femurs and tibiae with PBS, resuspended in medium containing GM-CSF at 2 × 105 cells/ml, and plated at a cell density of roughly 3.3–3.5 × 104 cells/cm2. Cells were cultured in standard 100-mm non-tissue culture-treated petri dishes, 100-mm Teflon dishes (Savillex), or 6-well plate Teflon inserts (Savillex) to reduce cell adherence (for 6-well inserts, BM cells were resuspended at 1.65 × 105 cells/ml). Cultures were grown for a total of 7–8 days to allow for sufficient DC differentiation from BM precursors. Various concentrations of E2, tamoxifen, and raloxifene were added alone or in combination from the first day of culture (day 0) and cells were fed every 3 days with medium containing GM-CSF. On day 3, cells were fed by adding a volume of fresh medium equivalent to that of the culture. For feeding on day 6, one-half of the culture supernatant was first removed and centrifuged to recover any cells before disposal. The cell pellet was then resuspended in an equal volume of fresh medium and added back to the original culture. At each feeding, E2, tamoxifen, and raloxifene were replenished at the indicated concentrations. For DC maturation, cultures were incubated for 12–14 h over a time period spanning days 6–7 or days 7–8 with 0.2–2.0 μg/ml LPS from Escherichia coli serotype O55:B5 (Sigma-Aldrich).

Flow cytometry

For flow cytometric analyses, murine DC cultures were analyzed on day 7 or 8 by three- or four-color surface staining. FACS staining buffer (PBS, 5% newborn calf serum, 0.1% sodium azide) was used for all washes and incubations. FACS staining was performed by standard procedures and is later described. Following harvest, 2 × 106 cells were incubated for at least 5 min with an unlabeled anti-CD11c/32 mAb (2.4G2) to block FcRs. Then surface staining was performed by incubating samples for 15 min with combinations of optimally titrated, fluorochrome, or biotin-conjugated mAbs specific for CD11c, CD11b, CD86, CD80, MHC class II, CD40, and...
TLC4. After one wash with 2 ml of buffer, allopbyocycin- or PerCP-conjugated streptavidin was added for another 15 min incubation to detect biotin conjugates. This step was followed by one wash with 2 ml of buffer. In most instances, cells were fixed with 1% paraformaldehyde before analysis with the addition being when anti-CD40 was included in staining combinations. Isotype control Abs tested by this staining procedure did not bind nonspecifically to cells from our cultures.

Unlabeled anti-CD11c/2, anti-MHC class II (Y3P) biotin, and anti-CD11c (N418) Alexa 488 were produced by the corresponding hybridomas (American Type Culture Collection), and were purified and labeled in our laboratory. Streptavidin-allophycocyanin, streptavidin-PerCP as well as the following mAbs: anti-CD80 biotin (16-10A1), anti-CD86 biotin and PE (GL1), anti-CD40 FITC (3/23), and anti-CD11b PerCP-Cy5.5 (M170) were from BD Pharmingen. Anti-CD11b PE (M1/70) and anti-TLR4 biotin (GL1) were from eBioscience. Anti-TLR4 PE (M1/70) was from BioLegend. Samples were run on a BD Biosciences FACSCalibur instrument capable of four-color detection and analyzed with CellQuest or FlowJo software.

OVA and dextran uptake

Chicken OVA labeled with Alexa 488 (OVA488) and dextran-labeled (10,000 m.w.) fluorescein (DexFITC) were obtained from Molecular Probes. The conditions for Ag uptake were chosen based upon methods used in similar studies (37, 38). At day 7, BMDC were harvested from DEF medium cultures and washed with PBS. Then 2 x 10^5 cells were resuspended in fresh DEF medium at a concentration of 1.5 x 10^6 cells/ml and were placed back into culture with either 50 μg/ml OVA488 or 100 μg/ml DexFITC for 30 min at 37°C. Ag uptake controls were conducted at 0°C with all other conditions being equal. The samples were then washed two times in FACS staining buffer and surface stained for CD11c, CD11b, and DextrFITC for 30 min at 37°C. Analysis was conducted with all other conditions being equal. The samples were then washed two times in FACs staining buffer and surface stained for CD11c, CD11b, and MHC class II as described and analyzed by FACs analysis. No E2, tamoxifen, raloxifene, ethanol, or DMSO was included in the medium during the 30 min Ag uptake.

Results

We investigated the effects of two clinically relevant SERM, tamoxifen and raloxifene, on DC development and expression of surface molecules important for APC function before and after activation by inflammatory stimuli. Based upon our and other previous observations regarding the inhibitory effects of tamoxifen on DC, we hypothesized that raloxifene would act similarly to block DC development by acting as an ER antagonist. The method of in vitro DC generation used in these studies involved a 7- to 8-day period of GM-CSF-supported BM culture in DEF or NORM medium. In contrast to medium containing regular FBS (NORM), DEF medium contained FBS stripped of steroid hormones and lacked the weakly estrogenic dye, phenol red. Use of DEF medium containing FBS stripped of steroid hormones resulted in dramatically reduced to 5% for CD11bhigh and 3% for CD11bblow cells (Fig. 1B). Because of the ability of tamoxifen and raloxifene to act as antagonists as well as agonists of ER in distinct cell types, it was possible that when added alone, either compound might have promoted DC differentiation relative to the control.

However, as shown in Fig. 1, neither tamoxifen nor raloxifene at 200 nM supported DC development in the absence of E2, suggesting that these SERM did not work as ER agonists on BMDC progenitors to promote DC differentiation.

Raloxifene and tamoxifen inhibit DC differentiation in the presence of E2

Normal levels of E2 in mouse serum vary based upon sex, the stage of the menstrual cycle, and pregnancy. Male mice possess lower levels of E2 (8–15 pg/ml) than females in diestrus (25–35 pg/ml) and in estrus (100–200 pg/ml) (39, 40). We performed competition experiments between E2 and SERM to measure effects on DC development. Based upon the results from our previous study of the E2 dependency of BMDC, we used E2 at either 0.1 or 1.0 nM (27 and 272 pg/ml), which are at its K<sub>p</sub> values for ER. These physiological amounts of E2 were efficient at supporting DC differentiation. We chose to use raloxifene from 1 to 200 nM and tamoxifen from 50 to 500 nM in our BM cultures because these concentrations are similar to the levels that have been detected in the plasma of humans taking SERM orally, 1.36 ng/ml (~2.5 nM) for raloxifene and 120 ng/ml (~200 nM) for tamoxifen (according to Physicians Desk Reference). The relative ER binding affinities, expressed as the percentage of E2 affinity for ER, have been estimated to be 34% for raloxifene (10) and 3% for tamoxifen (34) from competitive binding assays with ER<sup>–</sup> MCF-7 cells.

Results from a characteristic 8-day culture experiment are shown in Fig. 2. BM cultures were treated with vehicle only (a combination of ethanol, the vehicle for E2 and DMSO, the vehicle for SERM), E2 plus DMSO, or E2 plus variable doses of SERM. As before, it was found that in control cultures containing deficient medium not supplemented with E2 (Fig. 2A), few DC developed (9% CD11bhigh and 2% CD11bblow). However, addition of E2 alone (Fig. 2B) promoted the differentiation of both CD11bhigh (32%) and CD11bblow (30%) DC. Interestingly, tamoxifen and raloxifene inhibited the E2-mediated development of both of these DC populations, suggesting that these drugs competed with E2 for ER binding, and that excess ER-SERM complexes did not act similarly to ER-E2 complexes to promote DC differentiation. Although significant inhibition of CD11bhigh DC was observed, the most remarkable decreases were seen in the CD11bblow population.
nM tamoxifen (Fig. 2C) did not have an effect in competition with 1 nM E2 in this example, however, 500 nM tamoxifen (Fig. 2D), 50 nM raloxifene (Fig. 2E), and 100 nM raloxifene (Fig. 2F) all induced a dramatic reduction in DC. For example, CD11bhigh DC represented 32% of cultures given E2 alone; this was reduced to 24% by 500 nM tamoxifen (a 25% reduction) and 9–10% by 50–100 nM raloxifene (70% reduction). CD11bint DC in the same cultures were reduced from 30% with E2 alone, down to 13% for 500 nM tamoxifen (56% reduction) and around 4–5% for 50–100 nM raloxifene (85% reduction). As shown in this experiment, a third population of CD11c−CD11blow DC was repeatedly identified but was relatively unaffected by the presence or absence of E2. For this reason, CD11blow DC have been excluded from most of the subsequent analyses of DC subpopulations.

E2 often acts as a regulator of growth and differentiation. In general ovariectomy causes immune organs to enlarge whereas treatment with E2 results in significant shrinkage presumably due to the reported negative effects of E2 on lymphoid progenitors (41). Thus it was possible that ER antagonism by SERM might have led to an increase in numbers of undifferentiated lymphoid or myeloid progenitors in BM cultures. To confirm that the apparent decrease in the percentage of DC accurately reflects a real drop in DC numbers, we back calculated the total number of DC recovered from the cultures. Results from three independent experiments under various culture conditions are shown in Fig. 3. The results from Fig. 2 have been redisplayed in Fig. 3A, showing that SERM treatment of BM cultures in DEF medium supplemented with 1 nM E2

FIGURE 2. Tamoxifen and raloxifene competed with estrogen and inhibited the differentiation of DC from BM precursors. BM cultures were grown for 8 days in DEF medium supplemented with 1 nM E2 and various concentrations of SERM. At the end of the culture period, cells were harvested and stained with mAbs specific for CD11b and CD11c for FACS analyses. A, In the vehicle control (V) of ethanol and DMSO, few DC developed, especially the CD11bhigh DC. B, In the presence of 1 nM E2 plus DMSO, CD11bhigh and CD11bint DC development were both promoted. C, Addition of E2 plus 100 nM tamoxifen (T100) did not significantly affect DC development in this instance. D, Addition of E2 plus 500 nM tamoxifen (T500) reduced the frequency of both DC populations. Addition of E2 plus 50 nM raloxifene (R50) (E) or E2 plus 100 nM raloxifene (R100) (F) impaired DC development to a similar extent. Numbers indicate the percentage of total cells within the boxed region. The greatest effects were on the CD11bint DC population. Data are representative of 11 independent experiments.

FIGURE 3. The total number of DC in culture was reduced by exposure to tamoxifen or raloxifene. BM cultures were grown under several different conditions to assess SERM effects on DC differentiation. DC were differentiated in the presence of variable doses of tamoxifen (T), 4OH, raloxifene (R), or the DMSO vehicle (V). A, Differentiation for 8 days in DEF medium plus 1 nM E2 on nontissue culture-treated plastic petri dishes. Ethanol (EtOH), in which E2 was solubilized, and DMSO were added as a double vehicle control (V) to assess E2-independent DC differentiation. B, Differentiation for 7 days in DEF medium plus 0.1 nM E2 on Teflon. C, Differentiation for 7 days in NORM medium on nontissue culture-treated plastic. At the end of the culture period, cells were harvested, surface stained with mAbs specific for CD11b and CD11c, and analyzed by FACS. Total DC numbers were derived from cell counts and FACS plot data. The total number of cells recovered from one dish was very different among the three experiments depicted and is probably reflective of variations in culture conditions such as duration, type of plate used, and E2 concentration.
does indeed reduce the actual numbers of DC, CD11b<sup>high</sup> DC tended to be less dependent on E2 than CD11b<sup>int</sup> DC.

Only the nonadherent and loosely adherent cell fractions were analyzed in our initial studies (Fig. 3A) using petri dishes to which some DC adhered strongly, and previous experiments indicated that they were representative of the entire culture. To alleviate a potential sampling error, we began to use Teflon cultureware, which has been reported to result in significantly less cell adherence. A representative 7-day BM culture in Teflon with 0.1 nM E2 added to DEF medium is shown in Fig. 3B. These experiments demonstrated that tamoxifen and raloxifene had significant inhibitory effects under these conditions as well, and the increased sensitivity of the CD11b<sup>int</sup> population to SERM was still observed.

We also investigated the effects of a well-studied metabolite of tamoxifen, 4OH, in NORM medium, in which all the E2 present is derived from FBS. Upon direct comparison of the effects of 4OH to tamoxifen (Fig. 3C), we found 1 nM 4OH to be nearly as effective as 500 nM tamoxifen at inhibiting CD11b<sup>int</sup> DC development in NORM medium. The 1 and 10 nM 4OH and 50 nM tamoxifen slightly increased the number of CD11b<sup>high</sup> DC relative to the control but this effect was diminished when cultures were treated with 500 nM tamoxifen. The opposing effects of 50 and 500 nM tamoxifen on CD11b<sup>high</sup> DC in this system were an unexpected but reproducible finding for which we have no explanation. It is interesting to note that overall, there was less inhibition of DC differentiation by SERM in NORM medium cultures compared with DEF medium cultures that contained charcoal dextran stripped FBS, to which only E2 was added back. This implies that, in addition to E2, there are important factors influencing DC differentiation, which are removed by the charcoal dextran treatment process. Under all culture conditions, we found that the development of CD11b<sup>int</sup> DC was the most severely inhibited by SERM.

Raloxifene and tamoxifen augment Ag uptake by DC

Ag uptake capacity and surface expression of maturation markers are two parameters by which to gauge the maturation status of DC. Immature DC are efficient at Ag uptake and this function is reduced after maturation. We observed that immature DC from tamoxifen- and raloxifene-treated cultures had lower levels of MHC class II and CD86 compared with untreated DC suggesting that they are more immature (see discussion below and Fig. 6A). Therefore, we reasoned that a greater percentage of the DC that develop in the presence of SERM, relative to the control, might exhibit the ability to internalize high levels of soluble Ags. To test this, DC differentiated for 7 days in the presence of 0.1 nM E2, with or without one of the two SERM, were allowed to internalize OVA<sub>488</sub> or DexFITC for 30 min at 37°C. Ag incubations also were done at 4°C to allow for the distinction between signals from surface binding and internalization because uptake is arrested at low temperatures.

As expected, both raloxifene and tamoxifen augmented the uptake of OVA<sub>488</sub> and DexFITC by BMDC, defined in this study as all CD11c<sup>+</sup> cells (Fig. 4). There was a difference in Ag uptake capacity observed between the CD11b<sup>high</sup> and CD11b<sup>int</sup> cells; this is demonstrated in Fig. 5 from an independent repeat of the experiment. Immature CD11b<sup>high</sup> DC were found to be the most efficient at internalizing Ags in all treatments. This finding is consistent with the observation that CD11b<sup>high</sup> cells are relatively less mature based upon their overall lower expression of MHC class II and CD86 (31). However, the effect of both SERM to increase Ag uptake was most apparent in the CD11b<sup>int</sup> DC. The results were similar whether OVA<sub>488</sub> or DexFITC (data not shown) were used for uptake. These data indicate that SERM treatment of BM cultures during DC differentiation leads to DC that are functionally more immature.

Raloxifene and tamoxifen render DC hyporesponsive to LPS maturation

To determine whether the effects of tamoxifen and raloxifene on DC influence the final phase of differentiation, activation by inflammatory stimuli, we studied the effects of SERM exposure on expression of key surface markers important for DC to function as APC. As before, BM cultures were supplemented with 1 nM E2 and given vehicle or SERM. Relative to control immature DC, treatment of BM cultures with tamoxifen (500 nM) or raloxifene (100 nM) for 8 days led to reduced levels of MHC class II and CD86 on immature DC (Fig. 6A). We next tested whether these two SERM might prevent DC acquisition of a mature phenotype following activation. Therefore, we induced DC maturation with the bacterial cell wall component, LPS, and examined these cells.
for expression of MHC class II and CD86. Nearly 90% of control DC expressed high levels of MHC class II and CD86 after LPS exposure indicating that they had acquired a mature phenotype (Fig. 6B). When DC were treated with 500 nM tamoxifen, only 60% of the population expressed high levels of MHC class II and CD86 (Fig. 6B). Treatment with 100 nM tamoxifen did not significantly impair the expression of MHC class II and CD86 on immature or activated DC (data not shown). In the case of 100 nM raloxifene, only 42% of DC exhibited the mature phenotype (Fig. 6B). Hence, tamoxifen and raloxifene rendered DC hyporesponsive to LPS induced maturation. Effects were seen on both CD11b<sup>high</sup> and CD11b<sup>int</sup> DC, and as was observed during DC differentiation, phenotypic effects were often greater on the CD11b<sup>int</sup> DC population (data not shown).

We then tested titrated amounts of raloxifene, from 1 to 100 nM, against 0.1 nM E2 for two reasons: 1) to demonstrate that the observed effects were dose-dependent; and 2) to determine the inhibitory activity of doses similar to the low concentrations that have been measured in the plasma of patients undergoing raloxifene therapy (∼2.5 nM). We found that low doses of raloxifene were able to inhibit DC differentiation and maturation in response to LPS. Weak effects of raloxifene on CD40 and CD80 were observed at 10 and 100 nM although to a lesser degree than that observed for MHC class II and CD86 (Fig. 7A); only the results with the 100 nM concentration are depicted. Raloxifene significantly inhibited the expression of MHC class II and CD86 by LPS-activated DC at concentrations as low as 1 nM (Fig. 7B). In the control, 77% of DC expressed elevated CD86. This was decreased to 56, 31, and 35% of the population by 1, 10, and 100 nM raloxifene, respectively. Similarly, MHC class II was found to be elevated in 74% of mature DC in the control and this was reduced to 60% by 1 nM, 41% by 10 nM, and 45% by 100 nM raloxifene. In Fig. 7, the total percentage of DC that developed in cultures was reduced from a maximum of 74% in the control to 70, 59, and 56% by 1, 10, and 100 nM raloxifene, respectively (data not shown). These results demonstrate that low doses of raloxifene (10 nM) maximally impaired the ability of DC to mature in response to LPS.

**TLR4 expression is not reduced by raloxifene or tamoxifen**

DC express surface receptors including TLR family members that allow them to sense pathogens by recognizing molecules that contain pathogen associated molecular patterns. TLR4 is involved in the detection of bacterial LPS (42). We hypothesized that SERM-induced hyporesponsiveness to LPS is related to effects on TLR4.
expression levels. Interestingly, there were differences in the level of TLR4 expression between the CD11bhigh and CD11bin DC populations on control DC (Fig. 8A); CD11bhigh DC exhibited overall higher levels of TLR4 expression relative to CD11bin DC. There were slight differences in TLR4 expression following 100 nM raloxifene or 500 nM tamoxifen treatment. Exposure of DC to SERM did not significantly alter the levels of TLR4 on CD11bhigh DC (Fig. 8B). In the case of CD11bin DC, it was sometimes observed that SERM actually increased TLR4 expression to nearly the level seen on CD11bhigh DC (Fig. 8B). CD11bin DC were the most susceptible to influence by ER ligands, and if LPS hyporesponsiveness were due to reduced or absent TLR4 expression, we would expect to see lower levels on these cells. Our data indicate that raloxifene and tamoxifen do not impair the LPS-induced maturation of DC through modulation of TLR4 expression levels.

**Discussion**

We characterized the effects of the SERM, raloxifene and tamoxifen, on murine DC generated from GM-CSF-supported BM cultures, finding marked effects on both DC differentiation and DC activation by inflammatory stimuli. In experiments using medium lacking measurable E2, it was demonstrated that neither raloxifene nor tamoxifen alone, could act to promote DC development from BM precursors, indicating that they are not acting as ER agonists on these cells. As was shown previously, DC differentiation from precursors was maximal in the presence of E2, which seemed to act preferentially to support the growth of CD11c+CD11b+ DC. Simultaneous addition of raloxifene or tamoxifen from the first day of E2-supplemented culture greatly inhibited BMDC progenitor differentiation into CD11c+CD11b+ DC, in some experiments reducing DC numbers by as much as 85% relative to control cultures following exposure to 100 nM raloxifene. Less impressive yet still significant developmental inhibition of the CD11c+CD11bhigh DC subset was seen by both raloxifene and tamoxifen.

Oral delivery of SERM leads to their metabolism to chemical variants, some of which may exhibit differential potency as ER ligands. Metabolites of raloxifene are not known to be significantly active relative to the parent compound (43). In contrast, tamoxifen can be converted by liver enzymes to N-desmethyltamoxifen and the more potent form, 4OH, among other species (44–46). In agreement with other published observations, we found 4OH to be more potent than the parent compound. In our in vitro model, 1 nM 4OH was nearly as effective as 50 nM tamoxifen in inhibiting CD11b+ DC differentiation. Treatment of BM cultures with tamoxifen in our simplified culture system does not likely take into account the contributions of all active tamoxifen metabolites because it is unclear whether cells in BM cultures execute similar biochemical reactions. Further studies of SERM using in vivo models will be useful to better understand how they will ultimately affect immunity.

Although the numbers of DC in BM cultures exposed to SERM were reduced significantly, those DC that did develop displayed a phenotype indicative of an incompletely differentiated state. DC in raloxifene and tamoxifen treated cultures had reduced surface expression of both MHC class II and CD86 relative to control DC. This observation is consistent with other studies in our lab that have shown that acquisition of CD11c+ by cells in normal BM cultures may occur before expression of MHC class II; that is, one can detect a transitional population of CD11clow MHC class II−/+ cells distinct from the majority of CD11c+ MHC class II+ DC. The ability of DC to internalize molecules is an important aspect of Ag-presenting function that is reduced upon DC maturation. Consistent with maintenance of an immature phenotype, we found that raloxifene and tamoxifen increased the percentage of DC that internalized both OVA and dextran. Of all the DC populations we studied, CD11bin DC Ag uptake capacity was the most significantly augmented by SERM treatment relative to the control. The percentage of CD11bin DC that internalized OVA was increased 2.6-fold by 100 nM raloxifene and 3.3-fold by 500 nM tamoxifen treatment. We also demonstrated that raloxifene and tamoxifen inhibit DC activation in response to inflammatory stimuli. Both SERM rendered DC hyporesponsive to LPS maturation in a dose dependent manner, as measured by a significant impairment (30–50%) in the percentages of DC that up-regulated their expression of MHC class II and CD86. Less significant effects were seen for CD40 and CD80 on LPS-matured DC.
These results suggest that SERM influence DC in ways that can profoundly affect the performance of the immune system. By decreasing DC numbers, a possible consequence of SERM treatment in vivo may be the dampening of immune responses. The mechanism by which tamoxifen and raloxifene inhibit DC development from BM precursors is not known. Some clues may lie in the relationships between E2 and the transcription factor, NF-κB, and cytokines such as IL-6. Various subunits of NF-κB have previously been shown to be necessary for the in vivo development of DC in studies of mice (48–50). And according to one study, IL-6 diverted the differentiation of human monocytes to macrophages rather than to DC (51). Interestingly, E2-ER ligand complexes are reported to modulate both NF-κB activity (14) (52) and IL-6 (53), which implies that SERM might be able to inhibit DC differentiation by exerting effects on these factors.

Furthermore, NF-κB and IL-6, as well as other cytokines, cytokine receptors, and their downstream signaling molecules known to be important for APC function could be involved in SERM mediated suppression of DC maturation. A potential cytokine candidate could be IFN-γ because it increases the expression of MHC and costimulatory molecule expression on APC. BMDC from IFN-γ receptor knockout mice were found to be deficient in their ability to mature in response to LPS, much like SERM-treated DC (54), indicating that IFN-γ can serve as an autocrine maturation factor. The cytokine IL-12, produced by activated DC, also may be involved in such an autocrine fashion because it amplifies IFN-γ production (6). In contrast, the cytokine IL-6 is thought to inhibit DC maturation in a STAT3 dependent manner as shown by one study in which STAT3 activation in response to IL-6 was associated with preservation of BMDC in the immature state (55). There is evidence to suggest that estrogens control STAT3 activity (56, 57), although how this is accomplished and whether there is activation or suppression of STAT3 function may depend on tissue type, a common observation for the biological effects of ER. In one particular study of breast cancer cells, E2 suppressed the induction of STAT3 activity by IL-6 and this effect was overcome by tamoxifen (56). Thus, induction of LPS hypersponsiveness by raloxifene and tamoxifen may be due to their effects on IFN-γ, IL-12, and IL-6 production as well as STAT3 activity in BM cultures.

The ability of raloxifene and tamoxifen to impair DC maturation, by decreasing the percentage of DC expressing high levels of MHC class II and costimulatory molecules suggests that SERM-treated DC will be comparatively weak activators of T cells next to their untreated counterparts. Because costimulation regulates the extent of TCR signal amplification it is also possible that SERM treatment could result in greater numbers of tolerogenic DC. This idea is supported by data showing that exposure of T cells to immature DC displaying low amounts of Ag and costimulation leads to abortive T cell proliferation and tolerance (58). Furthermore, levels of displayed Ag and costimulation are reported to play a role in the complex process of DC-mediated naïve CD4+ Th cell polarization in vivo (59). In general, higher levels of peptide-MHC complexes and costimulatory molecule expression favor Th1 development whereas low levels are thought to result in Th2 development or perhaps no polarization at all if T cells are incompletely activated. Thus, the lower levels of these molecules found on SERM-treated DC might favor Th2 polarization of naïve CD4+ T cells. The hypothesis that SERM affect Th polarization is supported by a number of published reports indicating that estrogens can influence Th development during natural variation of hormone levels or in studies of autoimmune animal models (9). However, the data are conflicting perhaps due to differential dose effects of

To gain a better understanding of how raloxifene and tamoxifen make DC hypersensitive to LPS, we examined their expression of TLR4. The TLR4 and MD2 proteins together constitute the LPS receptor complex present at the cell surface (reviewed in Ref. 47). A third protein, CD14, also participates in LPS binding but functions primarily to deliver LPS to the receptor. Of these three molecules, only TLR4 has a cytoplasmic tail that harbors motifs allowing it to transmit intracellular signals. An important consequence of TLR4 signaling is the activation of NF-κB, which may be partially under the control of estrogens. We found that SERM did not down-regulate TLR4 expression on DC. Thus it will be of interest to determine whether raloxifene and tamoxifen affect MD2, CD14, or signaling molecules downstream of TLR4.
E2, as there is evidence that estrogens can promote either Th1 or Th2 development.

In conclusion, we have demonstrated that the clinically used SERM, raloxifene and tamoxifen, impair both the differentiation and LPS-induced maturation of DC. Our data raise concerns over the treatment of healthy individuals with SERM for prevention of breast cancer or osteoporosis. The data imply that SERM treatment of humans could result in suboptimal DC-mediated immune responses due to reduced DC numbers in vivo or decreased levels of MHC class II and costimulatory molecule expression displayed by DC. Considering the influence exerted by these factors on naive CD4+ T cell polarization, such phenotypic changes may lead to a Th2 bias. In cancer patients, SERM may interfere with immunotherapeutic strategies aimed at bolstering antitumor immune responses such as tumor Ag vaccination and promotion of DC differentiation and recruitment to tumors in vivo. Further studies of DC function after in vivo SERM exposure in animal models are needed and will be helpful in understanding the effects of SERM on the immune function of women receiving tamoxifen or raloxifene for breast cancer or osteoporosis prevention.

Disclosures

The authors have no financial conflict of interest.

References


