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Estrogen Receptor α Signaling in T Lymphocytes Is Required for Estradiol-Mediated Inhibition of Th1 and Th17 Cell Differentiation and Protection against Experimental Autoimmune Encephalomyelitis

Karine Lélù,*‡ Sophie Laffont,*‡ Laurent Delpy,§ Pierre-Emmanuel Paulet,*†‡ Therese Périnat,§ Stefan A. Tschanz,‖ Lucette Pelletier,*‡ Britta Engelhardt,¶ and Jean-Charles Guéry*‡†‡#

Estrogen treatment exerts a protective effect on experimental autoimmune encephalomyelitis (EAE) and is under clinical trial for multiple sclerosis therapy. Estrogens have been suspected to protect from CNS autoimmunity through their capacity to exert anti-inflammatory as well as neuroprotective effects. Despite the obvious impacts of estrogens on the pathophysiology of multiple sclerosis and EAE, the dominant cellular target that orchestrates the anti-inflammatory effect of 17β-estradiol (E2) in EAE is still ill defined. Using conditional estrogen receptor (ER) α-deficient mice and bone marrow chimera experiments, we show that expression of ERα is critical in hematopoietic cells but not in endothelial ones to mediate the E2 inhibitory effect on Th1 and Th17 cell priming, resulting in EAE protection. Furthermore, using newly created cell type-specific ERα-deficient mice, we demonstrate that ERα is required in T lymphocytes, but neither in macrophages nor dendritic cells, for E2-mediated inhibition of Th1/Th17 cell differentiation and protection from EAE. Lastly, in absence of ERα in host nonhematopoietic tissues, we further show that ERα signaling in T cells is necessary and sufficient to mediate the inhibitory effect of E2 on EAE development. These data uncover T lymphocytes as a major and nonredundant cellular target responsible for the anti-inflammatory effects of E2 in Th17 cell-driven CNS autoimmunity.


Multiple sclerosis (MS) is a T cell-mediated autoimmune disease characterized by the infiltration of inflammatory leukocytes, including macrophages and T cells, into the CNS, resulting in myelin damage (1). Clinical improvement in MS patients is commonly observed during pregnancy, suggesting that elevated levels of sex steroid hormones exert immunoregulatory activity, thereby inhibiting the autoimmune response and/or inflammation (2–4). Indeed, studies of estrogen’s effects on experimental autoimmune encephalomyelitis (EAE), a murine model of MS, have shown that 17β-estradiol (E2) administration inhibits disease development (5, 6). Likewise, a protective effect of estrogens has been reported in a pilot clinical trial using estradiol administration to MS patients (7, 8). These disease-modulating effects of exogenous estrogens in MS and its experimental model, EAE, may account for the beneficial effects of pregnancy on clinical symptoms in women with MS.

E2 actions are essentially mediated by two intracellular molecular targets: estrogen receptor (ER) α and ERβ. We and others have shown that the protective effect of E2 on EAE was mediated through ERα (9–11). E2-mediated EAE protection has been associated with immunoregulatory effects characterized by a strong inhibition of autoantigen-specific Th responses, including both Th1 (6, 9, 10, 12) and Th17 cells (13). Although it was initially thought that these anti-inflammatory effects of E2 were mediated through a direct action on T lymphocytes (12, 14), it has been subsequently suggested that myeloid cells, such as dendritic cells (DCs), rather than T cells, were the primary targets of estrogens responsible for the immunoregulatory effects of E2 leading to EAE protection (15, 16). Thus, despite the obvious impacts of estrogens on CNS autoimmunity, the ERα-expressing cell types that mediate the anti-inflammatory effect of E2 on EAE are not known.

In the present work, we sought to identify the cellular target of E2 in an experimental model in which E2 treatment induces complete and long-lasting EAE protection and is associated with a strong inhibition in autoantigen-specific Th1 and Th17 cell responses and CNS inflammation (6, 9, 10). To this end, we conditionally deleted ERα from the endothelial or the hema-
topoietic compartments, from macrophages, DCs, or T lymphocytes, and evaluated the protective effect of E2 on EAE in such mice. Despite the broad expression of EROs among immune cells, we unexpectedly found that the E2-mediated inhibition of Th17-driven CNS autoimmunity relies selectively on EROα expressed in T lymphocytes.

Materials and Methods

**Mice**

Female C57BL/6JRj (B6) mice were purchased from the Centre d’Elevage R. Janvier (Le Genest St. Isle, France) and maintained in our animal facilities under pathogen-free conditions. Mice with a disrupted *Esr1* gene, hereafter called EROα−/− (kindly provided by Pr P. Chambon and Dr. A. Krust, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France), have been previously described (17) and backcrossed into the B6 background for at least 10 generations. To generate mice lacking EROα in T and B cells, B6 EROα−/− were crossed to B6 Rag2−/− mice. We have recently generated mice selectively lacking EROa in hematopoietic/endothelial cells or myeloid cells by crossing mice carrying an *Esr1* gene in which exon 2 was flanked by loxP sites (17) (EROαfl/fl) with B6 mice expressing the Cre recombinase under the control of the Tie2 promoter-enhancer (18) or the lysozyme M (LysM) promoter (19). These mice have been described elsewhere (20–22). The targeted exon 2 of the *Esr1* gene sites encodes the first zinc finger of the DNA binding domain of EROα. Cre-mediated recombination of the conditional floxed allele leads to unambiguous inactivation of EROα (17). To generate mice selectively lacking EROα in DC or T lymphocytes, we crossed EROαfl/fl mice with mice expressing the Cre recombinase under the control of CD11c (23) or CD4 (24) promoter, respectively.

**Southern blot analysis**

Genomic DNA (10 μg) obtained from various tissues or from purified CD4+ T lymphocytes were digested with BamHI restriction enzyme (Invitrogen), electrophoresed on a 1% agarose gel, and transferred to positively charged nylon membranes (MP Biomedicals). A previously described EROa probe (P3') (17) was radiolabeled with (α-32P) dCTP (Amersham Biosciences). The membrane was hybridized overnight at 42°C then washed twice with 2× SSC, 0.1% SDS, for 10 min at room temperature and once with 0.1× SSC, 0.5% SDS, for 10–30 min at 52°C. Autoradiography was performed by exposing the blots to medical x-ray film (Agfa) at ~80°C for 1 to 2 wk. The 4.4-kb and 8.8-kb bands represent the targeted (floxed) allele and the deleted allele, respectively (17).

**Quantitative real-time PCR**

Purified CD4+ T cells were homogenized in TRIzol reagent (Invitrogen Life Technologies, San Diego, CA), and RNA was extracted following the manufacturer’s instructions. RNA samples (1 μg) were treated with DNase I (Invitrogen Life Technologies) and transcribed into cDNA using random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Reactions were performed in a 25 μl final volume using the SYBR Green PCR Master Mix (Eurogentec). Gene expression level was quantified using ABI Prism 7900 sequence detection system (Applied Biosystems). EROα-specific primer sequences have been described elsewhere (21). Results were analyzed using the SDS program, version 2.2 (Applied Biosystems). The relative expression of target transcripts in each sample was normalized to HPRT according to the ΔΔCt method.

**EAE induction and treatment**

For active EAE induction, mice were immunized s.c. in the flanks with 100 μg myelin oligodendrocyte glycoprotein (MOG)35–55 peptide (Neosystem, Strasbourg, France) emulsified in IFA supplemented with 4 mg/ml *Mycobacterium tuberculosis* (H37RA; Sigma-Aldrich, St. Louis, MO). Mice were injected i.p. with 200 μg pertussis toxin (Calbiochem, Darmstadt, Germany) at days 0 and 2 and examined daily for clinical signs of disease. The mice were scored as follows: 0, no detectable signs of EAE; 1, complete limp tail; 2, limp tail and hindlimb weakness; 3, severe hindlimb weakness; 4, complete bilateral hindlimb paralysis; 5, complete hindlimb paralysis and forelimb weakness; 5.5, total paralysis of both forelimbs and hindlimbs; and 6, death.

For E2 treatment, we used the standard protocol described by others (9, 10). Briefly, female mice were implanted s.c. in the scapular region with an E2 pellet (2.5 mg, 60-d release; Innovative Research of America) 7 d before immunization with MOG35–55 peptide as described above for EAE induction. Unless otherwise stated, 8–12-wk-old mice were used in all experiments. All the protocols used have been approved by our institutional review board for animal experimentation.

**T cell assays**

Mice were immunized in the flank with MOG35–55/CFP as for EAE induction. Seven to 12 d postimmunization, draining lymph nodes or spleens were harvested. For the recall assay with lymph node cells (LNC), total LNC were stimulated in vitro with the indicated concentrations of MOG35–55 peptide or Con A (Sigma-Aldrich) in HL-1 synthetic medium as described elsewhere (25). Splenic CD4+ T cells were purified using the Dynal mouse CD4 negative isolation kit (Invitrogen) and then cultured at 3 × 10^6 CD4+ T cells/well with irradiated syngeneic splenocytes (4 × 10^5 cells/well) in the presence of MOG35–55 peptide as previously described (11). Cultures were incubated for 3 d in a humidified atmosphere of 5% CO2 in air. For T cell proliferation assays, cells were pulsed during the last 8 h of culture with 1 μCi (37 KBq) [3H]ThdR (40 Ci/mmol; the Radiochemical Center, GE Healthcare, Little Chalfont, U.K.). Cells were harvested onto glass fiber filter membranes, and [3H]ThdR incorporation was measured by an MicroBeta Trilux luminescence counter (PerkinElmer, Waltham, MA). For cytokine analysis, sister cultures were harvested at 72 h. Supernatants were analyzed by ELISA for IFN-γ and IL-4 as described (26). IL-17 was measured by ELISA using specific mAb pairs from BD Pharmingen.

Flow cytometric analysis of CNS-infiltrating cells and intracellular cytokine staining

On the indicated day postimmunization, mice were anesthetized and perfused intracardially with ice-cold PBS. The brain and spinal cord were resected and then homogenized and digested for 30 min with collagenase D (Roche, Indianapolis, IN) and DNase I (Roche) under continuous agitation at 37°C. The cell suspension was then strained through a 70-μm nylon filter (Falcon) and washed with HBSS containing 20 mM HEPES. Cells were separated by centrifugation (2000 rpm for 30 min) on a discontinuous isotonic Percoll gradient containing 90% and 30% layers. Mononuclear cells at the 90–30% interface were collected, resuspended in HBSS-HEPES, and washed extensively in FACS buffer containing 1% FCS, 5 mM EDTA, and 0.1% NaN3 in PBS. For flow cytometry, cells were stained with PE anti-CD45.2, FITC-labeled anti-CD11b, and allophycocyanin–anti-CD4 mAbs, all purchased from BD Pharmingen. For intracellular cytokine staining, CNS-infiltrating cells were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich). Cells were cell surface stained with anti-CD4–APC, fixed, and permeabilized. Cells were stained intracellularly with FITC-anti-IFN-γ mAb and PE-conjugated anti-mouse IL-17, TNF-α, or IL-4 mAbs (BD Biosciences).

For intracellular cytokine staining of MOG-specific CD4+ T cells, purified splenic CD4+ T lymphocytes were stimulated for 18 h at 37°C with irradiated (1700 rad) syngeneic splenocytes with 10 μM MOG35–55 in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) for the last 4 h of culture. Cells were cell surface stained with anti-CD4 and anti-CD44 mAbs, fixed, and permeabilized. Cells were then stained intracellularly with FITC-anti-IFN-γ mAb and PE-conjugated anti-mouse IL-17, TNF-α, or IL-4 mAbs (BD Biosciences).

Data were collected on an FACS Calibur cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Generation of bone marrow chimeras**

Bone marrow (BM) cells from EROα−/−, EROα−/−, or CD4-EROαKO mice were injected into lethally irradiated (850 rad) wild-type (WT), EROαfl/fl Tie2Cre, or Rag2−/− EROα−/− B6 recipients. Eight weeks after reconstitution, mice were implanted with or not with 2.5 mg E2 pellet 1 wk before immunization with MOG peptide to induce EAE as described above.

**Histology, immunohistochemistry, and stereology**

Mice were anesthetized and perfused intracardiachially with PBS and then paraformaldehyde 2%. Intact spinal columns were removed and fixed in 4% buffered formalin. The spinal cords were dissected and embedded in paraffin before sectioning. Ten-micrometer-thick sections were stained with anti-CD45 mAb followed by HRP-labeled anti-rat IgG secondary Abs using standard procedure. The slides were counterstained with hematoxylin to visualize nuclei and analyzed by light microscopy.

Structural quantification of the infiltrates was done on ~8–10 random step sections cut perpendicular to the longitudinal spinal cord axis by means of an unbiased stereologic approach. The entire spinal cross sections were captured with a digital camera on a Nikon Eclipse E600.
microscope (Nikon) with a magnification of ×4, resulting in approximately four images per cross section. A test grid composed of lines and points was superimposed on these digital images using the STEPanizer stereology tool (27). Volume density (V/D) was assessed by counting test points and the count recorded with the STEPanizer. Each point represented an area on the section and therefore a volume (density) in three dimensions. The volume density was afterward correlated to the surface area density (S/D) of the spinal cord, assessed by counting intersections of a test line with the spinal profile border. S/D = I/L, in which I is the number of intersections with the border and L the length of the test lines. This relation resulted in an absolute metric value, V/D/S/D = V/L, with metric units mm^2/mm = μm indicating the thickness of a supposed layer of infiltrate around the spinal cord.

Statistical analysis

For comparison of EAE clinical scores between groups, repeated measures two-way ANOVA was performed followed by a Bonferroni post hoc test by using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Otherwise, pairwise comparisons between groups were conducted using the Mann–Whitney U test. All graphs show mean ± SEM.

Results

The anti-inflammatory effect of E2 on EAE is exclusively dependent on ERα and is lost in mice lacking ERα in both endothelium and hematopoietic cells

Using a well-established model of E2-mediated EAE protection (6, 9), we first evaluated the role of ERα using mutant mice exhibiting a fully disrupted Esrl gene (17, 28). Whereas administration of E2 1 wk before EAE induction markedly reduced disease incidence and severity in ERα−/− C57BL/6 mice, it had no effect on EAE development in ERα−/+ mice (Fig. 1A). These data confirmed previous works (9, 10) and establish that pharmacological activation of ERα by E2 is required to prevent EAE development in this experimental setting.

As a first step to identify the primary ERα-expressing cellular target responsible for the protective action of estrogens on EAE, we assessed the effect of E2 treatment on EAE development in Tie2-ERαKO mice in which the floxed ERα gene is conditionally invalidated in both endothelial and hematopoietic cells after crossing with Cre-expressing mice under the control of the Tie2 promoter (20, 21). As shown in Fig. 1B, disease course and severity were similar between Tie2-ERαKO mice and their ERαfl/fl littermate controls. Whereas E2 administration strongly inhibited disease development in ERαfl/fl mice, this treatment was ineffective in preventing EAE in Tie2-ERαKO mice (Fig. 1B).

Table I. The protective effect of E2 on active EAE is lost in mice lacking endothelium/hematopoietic ERα

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Incidence [no. (%)]</th>
<th>Death</th>
<th>Mean Day of Onset (± SEM)*</th>
<th>Mean Day of Peak (± SEM)*</th>
<th>Mean Maximal Score (± SEM)*</th>
</tr>
</thead>
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<tr>
<td>ERαfl/fl</td>
<td>7/7 (100)</td>
<td>0/7</td>
<td>12.7 ± 0.4</td>
<td>15.7 ± 0.4</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>ERαfl/fl + E2</td>
<td>0/7 (0)</td>
<td>0/7</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>Tie2-ERαKOfl/fl</td>
<td>6/6 (100)</td>
<td>0/6</td>
<td>13.3 ± 0.7</td>
<td>16.2 ± 0.5</td>
<td>4.4 ± 0.2 (NS)</td>
</tr>
<tr>
<td>Tie2-ERαKOfl/fl + E2</td>
<td>9/9 (100)</td>
<td>0/9</td>
<td>16.9 ± 0.8</td>
<td>19.4 ± 0.7</td>
<td>4.9 ± 0.1</td>
</tr>
</tbody>
</table>

Table summarizes data shown in Fig. 1.

*Disease mice.

*p < 0.01.
whereas no obvious differences were observed between control and E2-treated Tie2-ERαKO animals (Supplemental Fig. 1). Indeed, analysis of the phenotype of CNS infiltrates in diseased Tie2-ERαKO animals treated or not with E2 showed no overt change in the cellular profile of mononuclear cells with regard to the percentage of CD4+ T cells and macrophages or microglia (Supplemental Fig. 2C, 2D). Likewise, the frequency of pathogenic IL-17-producing CD4+ T cells (Supplemental Fig. 2E) was similar in both groups.

The protective effect of E2 treatment against EAE in control ERαfl/fl mice was associated with a strong inhibition of the proliferative response and the production of IFN-γ and IL-17 by splenic CD4+ T lymphocytes in response to MOG peptide (Fig. 1C). This immunoregulatory effect of E2 was lost in Tie2-ERαKO mice that developed similar proliferative responses and produced comparable amounts of both cytokines whether they were treated with E2 or not (Fig. 1C). These results strongly suggest that ERα activation in the endothelial and/or hematopoietic compartments downregulated EAE by limiting MOG-specific priming and differentiation of encephalitogenic Th1 and Th17 lymphocytes.

**ERα expression in hematopoietic cells is required for E2-mediated EAE protection**

To assess whether the protective effect of E2 on EAE was dependent on the expression of ERα in blood-derived hematopoietic cells, radiation BM chimeras were generated. Lethally irradiated WT B6 or Tie2-ERαKO recipients were reconstituted with BM cells from either ERα−/− or WT mice. Whereas E2-mediated EAE inhibition was maintained in control WT → WT or WT → Tie2-ERαKO BM chimeras (Fig. 2A), the protective action of E2 was lost in WT or Tie2-ERαKO mice reconstituted with ERα−/− BM cells (Fig. 2B). In this latter combination, despite some delay in disease onset in E2-treated mice, EAE then developed with similar incidence and severity as compared with untreated controls. Altogether, these data show that ERα expressed in hematopoietic cells rather than endothelial cells is required for the protective effect of exogenous E2 on EAE.

**ERα signaling in monocytes/macrophages or DCs is dispensable for E2-mediated EAE protection**

Hematopoietic cells, including encephalitogenic T cells, blood-derived macrophages, and DCs, are important triggers of inflammation during CNS autoimmunity (29, 30). To explore their respective roles in E2-mediated EAE protection, we first analyzed the effect of ERα inactivation in the APC compartment, including monocytes/macrophages and conventional DCs (cDCs). We recently generated mice selectively lacking ERα in monocyte/macrophages by crossing ERαfl/fl mice with mice expressing the
Cre recombinase specifically in the myeloid compartment, driven by the LysM promoter (22). LysM-ERαKO mice and their respective negative littermate controls (ERαfl/fl) developed clinical EAE with similar incidence, time of onset, and severity (Fig. 3A, 3B), indicating that ERα activation in monocytes/macrophages by endogenous estrogens had no measurable impact on the induction and progression of CNS autoimmunity. Administration of exogenous E2, however, prevented EAE development (Fig. 3A) and CNS inflammation (data not shown) with similar efficacy in both groups. Likewise, the proliferative response of MOG-specific T cell in the draining LNC as well as the production of IFN-γ and IL-17 were strongly inhibited in both E2-treated ERαfl/fl and LysM-ERαKO mice (Fig. 3C, 3D). Thus, these results show that ERα signaling in monocytes/macrophages was dispensable for the E2-mediated inhibition of encephalitogenic Th1/Th17 cell development and EAE protection.

Because it has been suggested that estrogens may program DCs to become tolerogenic (15, 31), we generated mice lacking ERα in the cDC compartment by crossing ERαfl/fl B6 mice with mice expressing the Cre recombinase under the control of a CD11c transgenic locus (23). Disease course and overall severity were similar between CD11c-ERαKO and ERαfl/fl littermate controls, showing that inactivation of ERα in the cDC compartment does not modify clinical EAE in female mice (Fig. 4A). Administration of E2 in CD11c-ERαKO resulted in a complete inhibition of EAE development over a 30-d observation period (Fig. 4B). These data, together with our data in the LysM-Cre system, indicate that ERα signaling in professional APCs, including monocyte-derived DCs

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Incidence [no. (%)]</th>
<th>Death</th>
<th>Mean Day of Onset (± SEM)</th>
<th>Mean Day of Peak (± SEM)</th>
<th>Mean Maximal Score (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERαfl/fl</td>
<td>10/10 (100)</td>
<td>4/10</td>
<td>14.1 ± 0.3</td>
<td>16.1 ± 0.4</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>ERαfl/fl + E2</td>
<td>2/7 (28)</td>
<td>0/7</td>
<td>30 ± 1*</td>
<td>ND</td>
<td>0.3 ± 0.2**</td>
</tr>
<tr>
<td>CD4-ERαKO</td>
<td>8/8 (100)</td>
<td>1/8</td>
<td>14.2 ± 0.8</td>
<td>14.6 ± 0.9</td>
<td>4.6 ± 0.3</td>
</tr>
<tr>
<td>CD4-ERαKO + E2</td>
<td>8/9 (88)</td>
<td>1/9</td>
<td>21.8 ± 1.3**</td>
<td>24.6 ± 1.5**</td>
<td>3.5 ± 0.6 (NS)</td>
</tr>
</tbody>
</table>

Table II. Sustained E2-mediated EAE protection is lost in mice lacking ERα in T lymphocytes

*Diseased mice.
*p < 0.05, **p < 0.01.

FIGURE 5. ERα signaling in T lymphocytes is required for the protective effect of E2 on EAE. ERαfl/fl mice were crossed to mice expressing Cre under the control of CD4 promoter to obtain CD4-ERαKO or control ERαfl/fl B6 mice. ERαfl/fl or CD4-ERαKO B6 female mice were left untreated (−) or implanted (+E2) with an E2 pellet 2.5 mg) 7 d before immunization with MOG35–55. A, Mice were assessed daily for clinical signs of disease. Data are expressed as mean ± SEM from 8–10 mice per group from two independent experiments. The effect of E2 treatment on disease course was evaluated by repeated-measures two-way ANOVA. Group differences in clinical score were tested at each time points using a Bonferroni post hoc test. B, Characterization of CD45+ infiltrates (shown as brown HRP reaction) by immunohistochemistry on spinal cord sections of ERαfl/fl and CD4-ERαKO mice on day 31. Scale bar, 100 μm. C, Relative CD45+ infiltrates in spinal cord sections were quantified by stereology and expressed as mean ± SEM of volume per surface area (V/S; μm) from five mice per group. D, LNC from CD4-ERαKO mice were recovered 13 d postimmunization and stimulated with MOG35–55 peptide. Recall proliferation and IFN-γ and IL-17 secretion in culture supernatants were assessed at 72 h. Data show mean ± SEM representative of three experiments with four to six mice per group. Statistical significance of difference between groups was analyzed using the Mann–Whitney U test. *p < 0.05, **p < 0.01.
and cDCs, is dispensable for the protective effect of E2 on EAE development.

**T lymphocytes are the main in vivo cellular targets of E2 mediating the inhibition of encephalitogenic CD4+ T cell priming and EAE protection**

Lastly, because E2 treatment had such a profound inhibitory effect on the priming of encephalitogenic CD4+ T cells, we assessed whether a T cell-intrinsic mechanism was involved. For this, we generated mice in which ERα was specifically invalidated in T cells (CD4-ERαKO) by crossing CD4-Cre transgenic mice with ERαfl/fl B6 mice. As expected, we observed complete Cre-mediated excision of the ERα gene in CD4+ T lymphocytes but not in nonlymphoid tissues (Supplemental Fig. 3). We next compared the effect of E2 treatment on EAE induction in CD4-ERαKO mice and their respective ERαfl/fl littermate controls. In the absence of E2 treatment, EAE developed with similar incidence, time of onset, and severity in both groups of mice (Table II). Whereas E2 treatment strongly inhibited EAE in control ERαfl/fl mice, long-term E2-mediated EAE protection was lost in the majority of CD4-ERαKO animals (Fig. 5A, Table II). Although there was still a significant delay in disease onset and the peaking of the disease (Table II) in E2-treated CD4-ERαKO mice, maximum disease scores were comparable to untreated CD4-ERαKO or ERαfl/fl mice (Table II). In agreement with clinical data, histological analysis of spinal cord sections of E2-treated CD4-ERαKO mice demonstrated strong CD45+ immune cell infiltrates in their CNS (Fig. 5B, 5C). Of note, stereological assessment of the CD45+ spinal cord infiltrates actually showed a significant increase in the number of inflammatory cells in the spinal cord of E2-treated CD4-ERαKO mice as compared with untreated controls (Fig. 5C). By contrast, E2-treated ERαfl/fl littermate controls, which were protected from EAE, were devoid of inflammatory infiltrates at the same time point (Fig. 5B, 5C). Analysis of the MOG-specific T cell responses in draining lymph nodes of CD4-ERαKO mice demonstrated that E2 treatment was unable to inhibit MOG-specific CD4+ T cell proliferation and development into IFN-γ- and IL-17-producing T cells if ERα was not expressed in T cells (Fig. S5).

In similar experiments, we also analyzed the frequency of MOG-specific splenic CD4+ T cells producing Th1 and Th17 effecter cytokines. Upon stimulation of purified CD4+ T cells with MOG35–55 peptide and irradiated syngeneic APC for 18 h, MOG-specific cytokine producing cells were detected among the CD4+CD44hi memory populations (Supplemental Fig. 4). In agreement with the data presented in Fig. 5, the percentages of MOG-specific CD4+ T cells producing IFN-γ, IL-17, or TNF-α were strongly reduced in E2-treated ERαfl/fl mice as compared with untreated controls (Fig. 6A). By contrast, E2 treatment was ineffective at preventing MOG-specific Th1 or Th17 cell differentiation in CD4-ERαKO mice (Fig. 6B). Altogether, these data show that E2 treatment inhibits the differentiation of MOG-specific CD4+ T cells into Th1 and Th17 cells in vivo through a mechanism that requires ERα expression in T lymphocytes.

To be able to pinpoint the cellular requirement for ERα signaling in the hematopoietic compartment, we next generated BM chimeras into Rag2−/−/ERα−/− B6 recipient mice. Irradiated Rag2−/−/ERα−/− mice were reconstituted with BM cells from ERαfl/fl, ERα−/−, or CD4-ERαKO mice. Mice that had received ERα−/− BM cells were still highly sensitive to E2-mediated EAE protection even in the complete absence of ERα in the host tissues (Fig. 7). In striking contrast, in mice reconstituted with BM cells from CD4-ERαKO mice, EAE development was unaffected by E2 treatment, and disease developed with similar time of onset, incidence, and severity as in untreated ERαfl/fl mice or E2-treated chimeras reconstituted with ERα−/− BM cells (Fig. 7). Altogether, these data firmly establish that activation of ERα by E2 in T lymphocytes is necessary and sufficient to mediate the inhibition of EAE development in the absence of extrahematopoietic ERα.

**Discussion**

Despite strong evidence that E2 or estriol have beneficial effects on the clinical signs of MS and EAE, little is known about the
E2-responsive target cells orchestrating the anti-inflammatory action of exogenous estrogens in peripheral lymphoid organs. Given that ER are expressed, though at different levels, in almost all tissues of the body, estrogen may influence CNS autoimmunity through its action on various cell types involved in multiple aspects of immune responses and inflammation. Indeed, it has been hypothesized that estrogen-induced protection in EAE could be mediated through several nonexclusive mechanisms, including direct or indirect effects on autoreactive T cells (6, 9, 10, 12), induction of Treg cells (14), modulation of APC functions (15, 16, 31), or direct neuroprotective effects in the CNS through ERα- and/or ERβ-mediated signaling in astrocytes (32, 33) or microglia (34, 35). In the present work, using newly created cell-type specific ERα-deficient mice in combination with BM chimera experiments, we demonstrate conclusively that ERα expressed in conventional T lymphocytes is the main mediator of the anti-inflammatory action of exogenous E2 on EAE through its capacity to inhibit Th1/Th17 priming in secondary lymphoid tissues.

These results are unexpected given previous results obtained by us and others that suggested that the anti-inflammatory action of E2 in EAE was not mediated directly by E2-responsive T cells, but rather through indirect effects on myeloid cells or other tissues (11, 15, 16). Indeed, in an adoptive EAE model, it was shown that ERα expression in encephalitogenic T cells was not required for E2-mediated EAE protection (16). Likewise, we previously reported that low-dose E2 administration could prevent acute EAE development in the absence of ERα expression in peripheral T cells (11). In this latter model, however, disease protection was not associated with immunomodulatory effects on autoantigen-specific CD4 T cell responses in vivo (11). Thus, differences in the dose of hormone and timing of administration may explain the discrepancies regarding the anti-inflammatory effects of E2 on T cell priming (6, 9, 11). Our data now demonstrate, in a well-established model of E2-mediated EAE protection (6, 9, 10), that ligand-inducible activation of ERα in T lymphocytes mediates the inhibition of Th1 and Th17 development, resulting in complete protection from CNS inflammation. This conclusion is supported by several lines of evidence. First, we established that hematopoietic ERα accounts for most of the anti-inflammatory effect of exogenous E2 on EAE and that E2 can operate in the complete absence of ERα in the host’s nonhematopoietic compartment. Second, by monitoring the E2 effect in conditional knockout mouse strains selectively lacking ERα in distinct immune cell populations, we provide evidence that ERα signaling was required in T lymphocytes, but not in monocytes/macrophages or DCs, for sustained inhibition of EAE. Lastly, in the absence of ERα in the host’s nonhematopoietic tissues, we showed that selective ERα inactivation in T cells abolished the protective effects of exogenous E2 on EAE development, thereby demonstrating the unique and nonredundant role of ERα signaling in T cells. Collectively, these data support the hypothesis that ERα signaling in T lymphocytes suppresses Th1/Th17 cell priming and CNS autoimmunity through a mechanism of immunoregulation that occurs in the secondary lymphoid tissues.

It has been suggested that estrogens may directly act on myeloid cells, such as DCs, to promote their development into tolerogenic DCs responsible for the anti-inflammatory effect of E2 in EAE (15, 31). Although we cannot exclude that these cells may be ultimately involved in the immunoregulatory mechanisms leading to inhibition of Th1 and Th17 cell priming in immune lymph nodes, our data now show that ERα expressed in monocyte/macrophages and cDCs does not represent the primary target of E2 in vivo. Additionally, E2 might act on nonhematopoietic cells, such as endothelial cells or CNS-resident cells (i.e., astrocytes), to reduce encephalitogenic T cell trafficking to the CNS and/or ongoing autoimmune inflammation (11, 32–34, 36). Although these mechanisms may cooperate together to limit and/or delay EAE development in some situations, they do not seem to be sufficient for full EAE protection in the absence of the T cell driven anti-inflammatory action of E2.

ERα not only function as ligand-activated transcription factors but can also mediate their effects through the rapid activation of cytoplasmic signal transduction pathways (37). Although ERα can mediate both classical genomic as well as rapid nongenomic responses to E2 (38), it has been recently suggested that the orphan G protein-coupled receptor 30 (GPR30) could be responsible for the mediation of membrane initiated nongenomic effects of E2 (39). A recent study in the EAE model has shown that administration of the GPR30 agonist G-1 conferred protection from EAE in WT but not in GPR30-deficient mice (40). However, a strong protective effect of E2 still persisted in GPR30-deficient mice, suggesting that ERα rather than GPR30 represented the main receptor targeted by E2 (40). Indeed, the role of GPR30 as a putative membrane ER has been recently challenged by studies demonstrating that GPR30 did not bind E2 (41) and was not necessary to induce the effects of estrogen in the reproductive tissues (42). Although it cannot be excluded that GPR30 and ERα could act in concert in some signal transduction pathways (43), the GPR30-dependent mechanisms of EAE protection could involve distinct cellular targets from those responsible for the suppressive effect of E2 on EAE.

In conclusion, our data emphasize a major role for ERα signaling in T lymphocytes in the anti-inflammatory effect of E2 and protection against CNS autoimmunity. We show that most of the anti-inflammatory effects of estrogens are due to the inhibition of encephalitogenic Th1 and Th17 cell development in lymph nodes through ERα expressed in T lymphocytes. Although the molecular and cellular mechanisms by which T cell intrinsic ERα signaling regulates Th1/Th17 differentiation remain to be characterized, our results add ERα to the growing list of nuclear receptors that have been shown to modulate Th cell differentiation (44–47). Understanding the cellular and molecular mechanisms by which ERα signaling in T lymphocytes mediates the anti-inflammatory effect of E2 may help to design selective ER modulators with T cell-specific agonist properties, but devoid of harmful effects on reproductive tissues, for specific intervention in Th1/Th17-mediated autoimmune diseases such as MS.

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Disclosures

The authors have no financial conflicts of interest.

References


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Supplemental Figure S1: The protective effect of E2 on CNS inflammation is lost in Tie2-ERαKO mice.

(A) ERαfl/fl control littersmates (n=7 mice per group) or (B) Tie2-ERαKO female mice (n=6 to 9 mice per group) were treated with E2 pellet (2.5 mg, 60 day release) one week before EAE induction by active immunization of with 100 µg MOG35-55 peptide in CFA. (A and B) Characterization of CD45+ infiltrates by immunohistochemistry on spinal cord sections of ERαfl/fl and Tie2-ERαKO mice sacrificed at day 28 (scale bar, 300 µm). (C and D) Relative CD45+ infiltrates in spinal cord sections were quantified by stereology and expressed as mean ± SEM of volume per surface area (V/S, µm) from 5 mice per group. Statistical significance of difference between groups was analysed using the Mann & Withney test (**, p < 0.01; ns, not significant).
Supplemental Figure S2: Analysis of CNS-infiltrating mononuclear cells in E2-treated Tie2-ERα<sup>KO</sup> mice. Female Tie2-ERα<sup>KO</sup> (5 and 4 mice per group) were implanted (open circles) or not (filled circles) with E2 pellets (2.5 mg, 60 day release) 7 days before immunization with MOG35-55. (A) Mice were assessed daily for clinical signs of disease. (B) Cumulative disease index (CDI), defined as the mean of the sum of the daily disease scores for the indicated period of time. At day 26 mice were killed and CNS infiltrating mononuclear cells from brain and spinal cord were enriched using Percoll gradient centrifugation and stained with anti-CD45, anti-CD4 and anti-CD11b mAbs. Frequencies of CD4<sup>+</sup> T cells (C) and CD45<sup>hi</sup> CD11b<sup>hi</sup> macrophages (D) were determined by flow cytometry. Data from individual mice are shown, and statistical significance of difference between groups was analyzed by using the Mann & Withney U test (ns, not significant). (E) CNS-infiltrating mononuclear cells were analyzed by flow cytometry for intracellular IL-17 expression in CD45<sup>+</sup> CD4<sup>+</sup> T cells after stimulation with PMA and ionomycin. Statistical significance of difference between groups was analysed using the Mann & Withney test (ns, not significant).
Supplemental Figure S3: Evidence for efficient deletion of ERα floxed allele in CD4+ T cells from CD4- ERαKO mice. (A) Restriction map of the floxed ERαfloxed targeting construct and the Cre-generated deletion allele. The Cre-mediated deletion event eliminates both the exon 2 of ERα and the additional BamHI site inserted together with the 5’ LoxP site. BamHI-digested DNA was hybridized with a specific DNA probe located downstream the exon 2 as indicated; 8.8 kb and 4.4 kb bands represent the deleted allele (KO) and the targeted allele (Floxed), respectively. (B) Southern blot analysis of genomic DNA obtained from CD4+ T cells or various tissues from CD4-ERαKO (Cre+) or littermate ERαfloxed controls (Cre−) mice as indicated (*, non-specific hybridization of the 3’ probe). (C) Purified lymph node CD4+ T cells were obtained from CD4-ERαKO or ERαfloxed mice treated or not with E2 and immunized with MOG35-55 in CFA. RNA was isolated from CD4+ T cells (purity >95%) and reverse transcribed. Real time PCR analysis of ERα transcripts was then performed and normalized to HPRT transcript abundance analyzed in parallel.
Supplemental Figure S4: Analysis of the cytokine production profile of MOG-specific splenic CD4+ T cells from control or E2-treated ERα^{fl/fl} or CD4-ERα^{KO} mice. Splenic CD4+ T cells were enriched by negative selection and assessed for intracellular expression of the indicated cytokines upon 18 hour activation with MOG35-55 peptide in the presence of irradiated syngeneic APCs. Brefeldine A was added for the last 5 hours of culture. Intracellular production of the indicated cytokines was assessed as described in Materials and Methods. Background cytokine production in the absence of peptide was < 0.02%.