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Neurobiology

T Lymphocytes Do Not Directly Mediate the Protective Effect of Estrogen on Experimental Autoimmune Encephalomyelitis

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Gender influences mediated by 17β-estradiol (E2) have been associated with susceptibility to and severity of autoimmune diseases such as diabetes, arthritis, and multiple sclerosis. In this regard, we have shown that estrogen receptor-α (*Esr1*) is crucial for the protective effect of 17β-estradiol (E2) in murine experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis. The expression of estrogen receptors among various immune cells (eg, T and B lymphocytes, antigen-presenting cells) suggests that the therapeutic effect of E2 is likely mediated directly through specific receptor binding. However, the target immune cell populations responsive to E2 treatment have not been identified. In the current study, we induced EAE in T-cell-deficient, severe combined immunodeficient mice or in immunocompetent mice with encephalitogenic T cells from wild-type *Esr1*+/+ or *Esr1* knockout (*Esr1*−/−) donors and compared the protective E2 responses. The results showed that E2-responsive, *Esr1*+/+ disease-inducing encephalitogenic T cells were neither necessary nor sufficient for E2-mediated protection from EAE. Instead, the therapeutic response appeared to be mediated through direct effects on nonlymphocytic, E2-responsive cells and down-regulation of the inflammatory response in the central nervous system. These results provide the first demonstration that the protective effect of E2 on EAE is not mediated directly through E2-responsive T cells and raise the alternative possibility that nonlymphocytic cells such as macrophages, dendritic cells, or other nonlymphocytic cells are primarily responsive to E2 treatment in EAE. *(Am J Pathol 2004, 165:2069–2077)*

A disproportionate number of women suffer from autoimmune diseases such as diabetes, arthritis, and multiple sclerosis.[1–3] Gender bias in autoimmune disease and other evidence suggests that sex steroid hormones play a role in regulating immune responses.[4,5] Immunomodulatory actions of estrogen appear to result from effects on immune cytokine production,[6,7] leukocyte adherence to vascular endothelial cells,[8–11] and impairment of macrophage function.[12–14] Additionally, and of relevance to central nervous system (CNS) autoimmune disease, a direct neuroprotective effect of E2 has also been demonstrated.[15–17] These effects of estrogen are mediated through specific receptors and depend on regulated expression and cellular distribution of these receptors.[18] We have shown recently that estrogen receptor-α (*Esr1*) is crucial for the beneficial therapeutic effect of 17β-estradiol (E2) in murine experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.[19] The expression of estrogen receptors among various immune cells (eg, T and B lymphocytes, antigen-presenting cells) suggests that the therapeutic effect of E2 is likely mediated directly through specific receptor binding. However, the target immune cell populations responsive to E2 treatment have not been identified. To understand the cellular basis of protective E2 responses,

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we induced EAE in lymphocyte-deficient severe combined immunodeficient (SCID) mice with encephalitogenic T cells from either wild-type (WT) *Esr1*+/+ or *Esr1*−/− (knockout) donors and compared the effects of E2 treatment. The results provide a demonstration of cellular requirements for *Esr1* expression and suggest that immune modulation of autoreactive T cells by E2 is indirect and is mediated through direct or indirect effects of E2 on CNS cells expressing the phenotypic characteristics of antigen-presenting cells.

Materials and Methods

Mice

Female, 10- to 12-week-old , C57BL/6 (*Esr1*+/+, WT) and C57BL/6J-Prkdc^{SCID} (*Esr1*+/+, SCID) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The mice were housed and cared for in the Veterinary Medical Unit of the Portland V.A. Medical Center according to institutional guidelines. B6.129 *Esr1*−/− (*Esr1*−/−, WT), estrogen receptor-α knockout mice were provided by Dr. Paul Cooke (University of Illinois, Urbana, IL).

Treatment of Mice with 17-β-Estradiol

For 17β-estradiol (E2) hormone therapy, a single 3-mm pellet containing 15 mg of E2 (Innovative Research of America, Sarasota, FL) was implanted (subcutaneously) on the back of each mouse 14 days before induction of EAE. The pellets provide continuous controlled release of a constant level of hormone throughout a period of 60 days. Control animals were left untreated. Preliminary experiments showed unequivocally that pellets containing saline did not affect the course of EAE (unpublished data). The expected serum concentration of 17-β-estradiol was verified in all mice from each group by radioimmunoassay.

Adoptive Transfer of EAE

To prepare T-cell lines specific for MOG-35-55 peptide, C57BL/6 mice were immunized subcutaneously at four sites above the flanks with a total of 0.2 ml of an emulsion containing 200 μg of MOG-35-55 peptide in complete Freund's adjuvant containing 200 μg of heat-killed *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI). Ten days after immunization, a single cell suspension of lymph node and spleen cells was cultured with MOG-35-55 peptide (20 μg/ml) at 8×10^6 cells/ml in stimulation medium (RPMI 1640 medium supplemented with nonessential amino acids, sodium pyruvate, 2-ME, and 10% fetal bovine serum) for 48 hours. Antigen (Ag) specificity of the T cells was evaluated *in vitro* by a thymidine incorporation proliferation assay after stimulation with MOG-35-55 peptide. For adoptive transfer, 20×10^6 cells per mouse were transferred by intraperitoneal injection. On the same day and 2 days after the cell transfer, mice were injected (intraperitoneally) with pertussis toxin (75 and 200 ng/mouse, respectively; List Biological Laboratories, Campbell, CA). Additionally, a portion of the T cells was expanded for 5 to 10 days after the first stimulation in medium containing interleukin (IL)-2 (100 U/ml). These cells were cultured with anti-mouse CD3 (1 μg/ml; clone 145-2C11; BD PharMingen, San Diego, CA) and anti-CD28 (1 μg/ml; clone 37.51; BD PharMingen) monoclonal antibodies immobilized on a tissue culture plate with IL-12 (20 ng/ml; R&D Systems, Minneapolis, MN) and IL-18 (25 ng/ml; MBL, Nagoya, Japan) added to the culture medium for 24 hours. After this time cells were washed three times with phosphate-buffered saline (PBS) and transferred intraperitoneally into naive B6 recipients (1 to 4×10^6 cells/mouse). Mice were injected with pertussigen on days 0 and 2 as above.

Evaluation of Clinical Severity

Mice were scored daily for development of EAE according to the following scale (0 to 6): 0, no signs; 1, limp tail; 1.5, moderate hind limb weakness with difficulty to right itself; 2, moderate hind limb weakness without ability to right itself; 2.5, moderate hind limb weakness without ability to right itself; 3, moderately severe hind limb weakness, walks upright only a few steps; 3.5, moderately severe hind limb weakness, paralysis of one limb; 4, severe hind limb weakness; 4.5, severe hind limb weakness with mild fore limb weakness; 5, paraplegia with no more than moderate fore limb weakness; 5.5, paraplegia with severe fore limb weakness (quadriplegia); 6, moribund condition.

Flow Cytometry

Three-color (fluorescein isothiocyanate, phycoerythrin, cychrome) fluorescence flow cytometry analyses were performed to determine the phenotypes of cells isolated from brain and spinal cord. Briefly, cells were washed with staining medium (PBS containing 0.1% NaN_3 and 2% fetal calf serum) and preincubated with anti-mouse CD16/CD32 monoclonal antibody to block nonspecific binding to Fc receptors. All antibodies were purchased from PharMingen. Pooled cells were divided equally (1 to 5×10^5 cells per tube) and were stained with a combination of the following monoclonal antibodies: rat anti-mouse CD3, CD4, CD8, CD11b, CD11c, CD45, VLA-4, LFA-1 for 25 minutes on ice. After incubation cells were washed three times with staining medium and analyzed immediately with a FACScan using CellQuest (Becton-Dickinson, Mountain View, CA) software. Data represent 10,000 events.

Histological Analysis of Spinal Cords

Mice were randomly selected from each E2- and placebo-treated group. Spinal cords were isolated and fixed in 10% paraformaldehyde. Transverse paraffin sections of the spinal cords were stained with Luxol Fast Blue-periodic acid-Schiff reagent-hematoxylin (LFB-PAS-H). The slides were analyzed by light microscopy. Demyelination was detected in spinal cord sections as a decrease or

Table 1. Susceptibility to Passively Transferred EAE Using *Esr1*+/+ and *Esr1*−/− Encephalitogenic T Cells among Untreated and E_2-Treated *Esr1*+/+WT, *Esr1*−/−WT, and *Esr1*+/+ SCID Recipients

Treatment	Incidence	Onset*	Peak*	CDI*	E_2 (pg/ml)	Thymic Index (mg/g)	Uterine Index (mg/g)
Esr1+/+T cells → *Esr1*+/+WT							
Placebo	5/7	6.6 ± 1.2	4.7 ± 0.2	38.1 ± 11.7	36.8 ± 10.3	4.2 ± 1.1	5.6 ± 0.2
E_2	0/5†	–†	0†	0†	4,171.5 ± 378.8†	0.9 ± 0.3†	12.0 ± 0.3†
Esr1+/+T cells → *Esr1*−/−WT							
Placebo	4/5	22.0 ± 2.31	4.0 ± 1.22	17.2 ± 13.7	25.0 ± 0.0	2.4 ± 0.5	6.8 ± 0.7
E_2	4/5	20.0 ± 0.0	3.7 ± 1.8	21.0 ± 11.5	4,384 ± 210.4‡	1.3 ± 0.2‡	4.9 ± 0.9
Esr1+/+T cells → *Esr1*+/+SCID							
Placebo	10/11	15.3 ± 2.1	4.3 ± 0.7	27.0 ± 4.9	27.0 ± 3.4	2.8 ± 0.9	5.9 ± 0.6
E_2	1/7§	20§	1§	1§	4,900 ± 297§	2.0 ± 0.3	13.6 ± 6.6§
Esr1−/−T cells → *Esr1*+/+SCID							
Placebo	9/12	0.5 ± 1.7	2.7 ± 0.9	21.0 ± 7.0	23.6 ± 1.7	2.1 ± 0.5	6.3 ± 0.9
E_2	0/13¶	–¶	0¶	0¶	5,253 ± 93.5¶	1.7 ± 0.4	13.2 ± 4.2¶

*Onset, peak, and CDI are presented as the mean trait value ± SEM. The CDI was determined by summing the daily clinical scores.
† *P* value ≤ 0.05, as compared to *Esr1*+/+ T cells → *Esr1*+/+ WT without E2.
‡ *P* value ≤ 0.05, as compared to *Esr1*+/+ T cells → *Esr1*−/− WT without E2.
§ *P* value ≤ 0.05, as compared to *Esr1*+/+ T cells → *Esr1*+/+ SCID without E2.
¶ *P* value ≤ 0.05, as compared to *Esr1*−/− T cells → *Esr1*−/− SCID without E2.

loss of blue stain from the white matter. Inflammatory cells were detected as an accumulation of darkly stained (hematoxylin stained) nuclei.

Reverse Transcriptase-Polymerase Chain Reaction

For quantitative real-time polymerase chain reaction analysis, total RNA was extracted from spleen and spinal cord using Total Rneasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and cDNA was prepared with 2.5 μmol/L of random hexamer primers. The sequence-specific primers were designed using Primer Express software (Applied Biosystems, Inc., Foster City, CA).[20] The levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-β, RANTES, MIP-2, IP-10, CCR1, CCR2, CCR6, CCR7, and CCR8 expression were quantified by real-time polymerase chain reaction using the ABI 7000 sequence detection system (Applied Biosystems, Inc.). Amplification was performed in a total volume of 25 μl for 40 cycles and products were detected using SYBR Green I dye (Molecular Probes, Eugene, OR). Samples were run in triplicate and their relative expression levels were determined by normalizing expression of each target to L32. Expression levels of normalized samples are displayed in relative expression units.

Results

Passive Transfer of EAE Using Either Esr1+/+ *or* Esr1−/− *MOG-Specific T Cells Is Inhibited in SCID Mice Pretreated with 17β-Estradiol*

A role for myelin antigen-specific T lymphocytes in the etiology of multiple sclerosis has been appreciated for many years[21,22] and expression of *Esr1* by these cells raises the question as to whether they are the primary E2-responsive cells in EAE. To evaluate this possibility, we induced EAE with E2-reposnsive *Esr1*+/+ encephalitogenic T cells in immunocompetent E2-responsive mice (Table 1, *Esr1*+/+ T cells → *Esr1*+/+ WT), in immunocompetent E2-nonresponsive mice (Table 1, *Esr1*+/+ T cells → *Esr1*−/− WT), and in E2-responsive T-cell-deficient SCID mice (*Esr1*+/+ T cells → *Esr1*+/+ SCID; Table 1). We also induced EAE with E2-nonresponsive *Esr1*−/− encephalitogenic T cells in E2-responsive T-cell-deficient SCID mice (Table 1, *Esr1*−/− T cells → *Esr1*+/+ SCID). E2 responses for each donor/recipient combination were detected by comparing E2-treated and untreated (placebo) animals (Table 1).

As expected, *Esr1*+/+ animals with EAE induced by *Esr1*+/+ T cells (Table 1, *Esr1*+/+ T cells → *Esr1*+/+ WT) were highly susceptible to treatment with E2, because E2-treated animals had reduced incidence (none of five versus five of seven), reduced peak disease severity (0 versus 4.7 ± 0.2) and reduced CDI (0 versus 38.1 ± 11.7). Measures of serum E2, thymic index, and uterine index demonstrated that disease differences were associated with differences in systemic levels of E2. In contrast to the *Esr1*+/+ WT mice with EAE induced by *Esr1*+/+ T cells, *Esr1*−/− WT mice with EAE induced by *Esr1*+/+ T cells (Table 1, *Esr1*+/+ T cells → *Esr* −/− WT) developed severed EAE that did not respond to E2 treatment, indicating that *Esr1* expression by transferred encephalitogenic T cells was not sufficient and indicating that *Esr1* expression by recipient-derived cells was necessary for the E2 response in these immunocompetent mice.

To evaluate the role of E2-responsive recipient-derived cells, the E2 response was evaluated in placebo- and E2-treated immunodeficient *Esr1*+/+ SCID mice with EAE induced by *Esr1*+/+ T cells (Table 1, *Esr1*+/+ T cells→ *Esr1*+/+ SCID). Similar to immunocompetent *Esr1*+/+ WT mice, T-cell-deficient *Esr1*+/+-immunodeficient SCID mice were also susceptible to treatment with E2, displaying an absence of disease compared to pla-

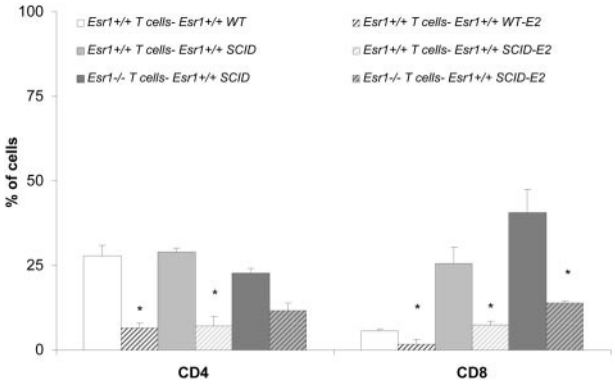

Figure 1. E2 treatment decreased the frequency of encephalitogenic T cells in the CNS of *Esr1*+/+ WT and *Esr1*+/+ SCID recipients. MNCs were isolated from brain and spinal cord harvested 10 days after disease onset from five mice. Cells were stained with anti-mouse CD3 and anti-mouse CD4 and anti-mouse CD8. Data presented are mean from two independent experiments and indicate the percentage of total gated cells that were dual-positive for CD3 and CD4 or CD8. Significance between control and experimental groups were determined by Student's *t*-test (*, $P < 0.05$).

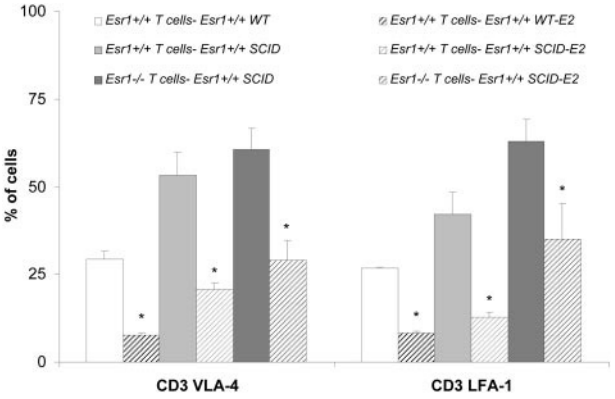

Figure 2. E2 treatment decreased the frequency of adhesion molecules on encephalitogenic T cells in the CNS of C57BL/6 and C57BL/6 SCID recipients. MNCs were isolated from brain and spinal cord harvested at 10 days after onset from five mice. Cells were stained with anti-mouse CD3 and anti-mouse VLA-4 and anti-mouse LFA-1 to identify expression of these adhesion molecules on T cells. Data presented are percentage of total gated cells that were dual-positive for CD3 and VLA-4 or LFA-1. Significance between control and experimental groups were determined by Student's *t*-test (*, $P < 0.05$).

cebo-treated mice. *Esr1*+/+ T-cell-deficient SCID mice with EAE induced by *Esr1*−/− T cells (*Esr1*−/− T cells → *Esr1*+/+ SCID; Table 1) also responded to E2 compared to placebo with complete absence of disease, demonstrating that *Esr1* expression by pathogenic T cells was not necessary for the E2 protection even in the absence of all other T cells. As expected, all E2-treated *Esr1*+/+ mice had a significant, nearly threefold increase in uterine index weight [expressed as uterine weight (mg) divided by body weight (g)] compared to untreated mice. We observed the typical decrease in thymic index after E2 therapy in C57BL/6 (*Esr1*+/+ WT) mice and a more modest lowering in the thymic index in E2-treated SCID mice compared to placebo-treated mice, apparently because of an overall reduced thymic index in this immunodeficient strain. Radioimmunoassay measurement of E2 serum levels in mice implanted with 15-mg pellets showed the mean level ranged between 4000 to 5000 pg/ml, which was significantly higher than in placebo-treated mice (Table 1). Taken together, these results are consistent with previous findings[19] regarding the requirement for E2 signaling through *Esr1* and confirm that E2 protection in EAE does not require direct E2 signaling to pathogenic T cells expressing the estrogen receptor-α.

E2 Blocks Trafficking of MOG-Specific T Cells into CNS

In addition to E2-mediated effects on clinical disease, we evaluated the effect of E2 on T-cell trafficking to the CNS. As shown in Figure 1, E2 treatment caused a reduction of inflammatory T cells in C57BL/6 and C57BL/6 SCID recipients of *Esr1*+/+ cells, with a marked reduction in $CD4^+$ T cells in *Esr1*+/+ recipients as determined by fluorescence-activated cell sorting analysis. In C57BL/6 SCID recipients of *Esr1*−/− cells, we observed a modest decrease in $CD4^+$ T cells and a substantial drop in CD8 cells in the CNS. The percentage of $CD8^+$ T cells was substantially higher in CNS of untreated SCID recipients of *Esr1*+/+ or *Esr1*−/− cells when compared to untreated C57BL/6 recipients of *Esr1*+/+ T cells. This was most likely because of expansion of these cells within the SCID environment, as was reflected by the higher number of cells recovered per cord. However, the frequency of $CD8^+$ cells in the CNS was significantly reduced after E2 therapy in each transfer group. Moreover, the number of $CD3^+$ T cells co-expressing inflammatory adhesion markers VLA-4 and LFA-1 in the brain was reduced in E2-protected versus sick mice (Figure 2), indicating that E2 treatment blocks entry of T cells into the CNS indirectly and independent of E2 effects on adhesion molecule expression by the transferred encephalitogenic T cells. Although some of these changes did not achieve statistical significance, the lack of *Esr1* on encephalitogenic T cells did not abrogate the protective effect of E2. Histological sections from brain of C57BL/6 (Figure 3, A and B) and C57BL/6 SCID (Figure 3, C and D) recipients of *Esr1*+/+ cells showed dramatic differences in pathological disease comparing E2-treated versus placebo-treated mice. C57BL/6 SCID recipients of *Esr1*−/− MOG-specific T cells also showed dramatically decreased severity of pathological disease with E2 treatment (Figure 3; E, F, K, L).

E2 Decreases CNS Macrophages and Dendritic Cells and Increases CNS Microglia

Resident microglia and/or infiltrating macrophages participate in disease pathogenesis through effects on blood brain barrier permeability, antigen presentation, immune regulation, and elaboration of cytotoxins (reactive oxygen and nitrogen, TNF-α).[23–25] Because these cells express *Esr1* in all of the E2-responsive transfer combinations used in this study, disease-modulating E2 effects on these cells were likely.[26] To evaluate this possibility, cells isolated from the CNS were triple-stained with CD11b, CD11c, and CD45. The CNS of E2-treated mice contained fewer harvested mononuclear cells and fewer

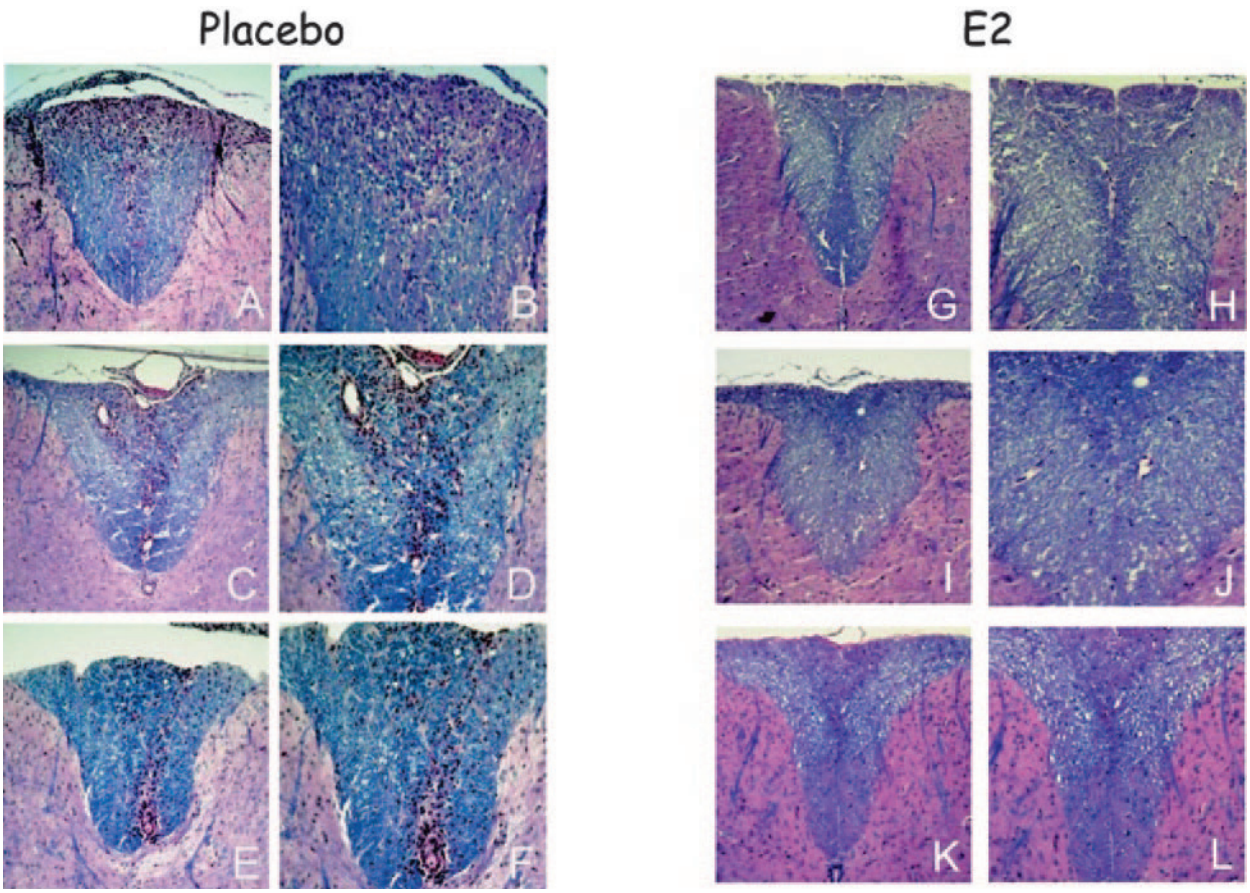

Figure 3. Fixed paraffin-embedded spinal cord sections from control (**A–F**) or E2-treated (**G–L**) recipients. Spinal cord from control *Esr1*+/+ T cells → *Esr1*+/+ WT (**A**, **B**), *Esr1*+/+ T cells → *Esr1*+/+ SCID (**C**, **D**), and *Esr1*−/− T cells → *Esr1*+/+ SCID recipients showed dense mononuclear infiltration with apparent loss of myelin (blue stain, luxol fast blue) in the surrounding myelinated tissue. Spinal cord from E2-treated control *Esr1*+/+ T cells → *Esr1*+/+ WT (**G**, **H**), *Esr1*+/+ T cells → *Esr1*+/+ SCID (**I**, **J**) showed no visible signs of inflammation whereas in spinal cord of *Esr1*−/− T cells → *Esr1*+/+ SCID (**K**, **L**) single mononuclear cells were present without visible demyelination. Original magnification: ×50 (**A**, **C**, **E**, **G**, **I**, **K**); ×150 (**B**, **D**, **F**, **H**, **J**, **L**).

$CD11b^+$ $CD11c^+$ dendritic cells compared to untreated mice and this did not depend on T cell's expression of *Esr1* (Table 2). The CNS from untreated *Esr1*−/− T cells → *Esr1*+/+ SCID mice had the most total CNS mononuclear cells (40×10^4 per cord) and the highest proportion of $CD11b^+$ $CD11c^+$ dendritic cells (15 ± 3%), indicating that cellular infiltration into the CNS and CNS APC activity were vigorous during disease induced in SCID mice by

Table 2. The Frequency of DCs, Peripheral Macrophages, and Resident Microglia in CNS of Sick and Protected Mice

	Esr1+/+ T cells → *Esr1*+/+ WT		*Esr1*+/+ T cells → *Esr1*+/+ SCID		B6 129 *Esr1*−/− T cells → *Esr1*+/+ SCID	
	No treatment (MFI)*	Treatment (MFI)	No treatment (MFI)	Treatment (MFI)	No treatment (MFI)	Treatment (MFI)
Total cells harvested per mouse	30×10^4	5×10^4	24×10^4	4×10^4	40×10^4	6×10^4
Percentage of CD11b+CD11c+ (DC) among total	6 ± 1	2 ± 1	9 ± 2	3 ± 2	15 ± 3	4 ± 2
Percentage of CD45+CD11b− (T cells) among total	76 ± 5	22 ± 6	72 ± 4	18 ± 5	74 ± 4	44 ± 10
Percentage of CD45+CD11b+ (Mø) among total	24 ± 4	78 ± 3	18 ± 2	72 ± 7	26 ± 3	56 ± 5
Percentage of CD45+ among CD11b+	100	100	100	100	100	100
Density of CD45 expression						
CD45intr (microglia)	30 ± 5 (671)	69 ± 2 (560)	23 ± 2 (790)	84 ± 6 (351)	35 ± 7 (897)	72 ± 4 (585)
CD45high (Mø)	70 ± 2	31 ± 1	77 ± 8	16 ± 8	65 ± 9	28 ± 2

MNCs were isolated from brain and spinal cord harvested at 10 days after disease onset from five mice. Cells were stained with anti-mouse CD11b, CD11c, and CD45. Data show the percentage of total gated cells that were positive for the indicated markers. The frequency of CD11b with different densities of CD45 were estimated among $CD11b^+$ $CD11c^-$ (R2) as described in Material and Methods. The values presented are representative of one of two independent experiments.

*Mean fluorescence intensity (MFI) for CD45 as measured among $CD11b^+$ (R3).

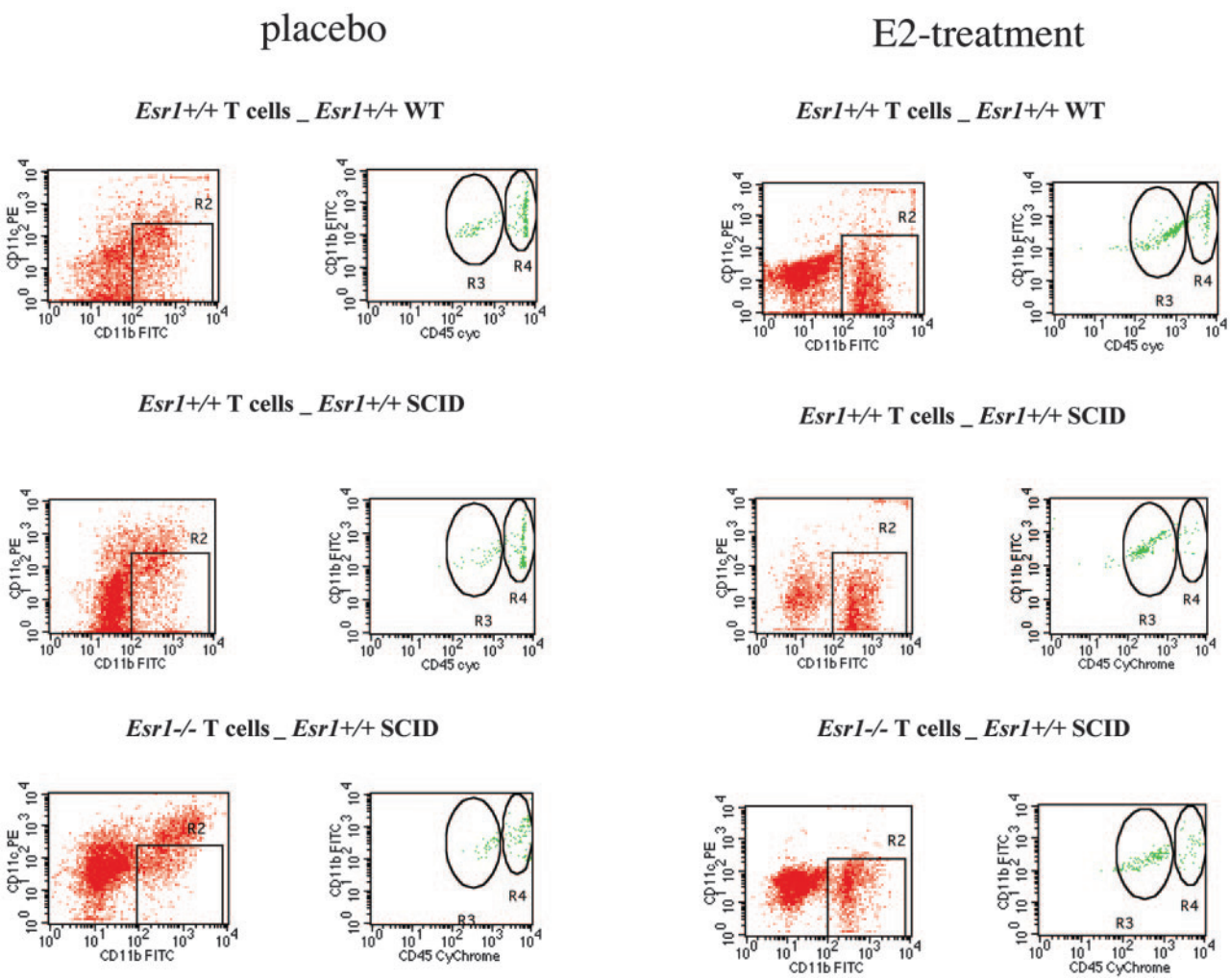

Figure 4. CD45 expression of microglia/CNS-associated macrophages from control and E2-treated recipients. Microglia/CNS-associated macrophages were gated as $CD11b^{+}CD11c^{-}$ cells (R2) to exclude the potential dendritic cells. All cells from R2 express CD45 and subdivide into two main populations: $CD45^{intr}$ (R3) and $CD45^{high}$ (R4).

Esr1−/− or *Esr1*+/+ T cells in the absence of E2 treatment. $CD45^{+}$ $CD11b^{-}$ cells comprised primarily of T lymphocytes were similar among all untreated mice and were markedly decreased after E2 treatment. It is notable that the decrease in $CD45^{+}$ $CD11b^{-}$ cells was not as great (74 ± 4, untreated; 44 ± 10, E2 treated) in mice with *Esr1*−/− T cells compared to either WT (C57BL/6) (76 ± 5, placebo; 22 ± 6, E2 treated) or SCID mice (72 ± 4, placebo; 18 ± 5, E2 treated) with *Esr1*+/+ T cells, suggesting a possible role for T cell *Esr1* in the loss of T cells from the CNS of E2-treated mice.

In addition to the loss of dendritic cells and T cells with E2 treatment, analytical-negative gating of the $CD11c^{+}$ cells (Table 2; Figure 4, R2 gate) permitted analysis of the $CD11b^{+}$ $CD11c^{-}$ cells (microglia and macrophages) for expression of CD45, a marker for distinguishing $CD11b^{+}$ resident microglia from infiltrating macrophages. In contrast to the decreases in total cells, dendritic cells, and T cells induced by E2, total $CD45^{+}$ $CD11b^{+}$ myelomonocytic cells increased with E2 treatment, reflecting a higher proportion of microglial cells because of decreased total inflammatory cells. This was also demonstrated by higher proportions of $CD11b^{+}$ $CD45^{intr}$ cells (microglia) (Table 2; Figure 4, R3 gate) in E2 compared to untreated mice. As was found for dendritic cells, E2-treated mice had relatively fewer $CD11b^{+}$ $CD45^{high}$ cells (macrophages) (Table 2; Figure 4, R4 gate) in the CNS compared to untreated mice and these differences were observed independent of *Esr1* expression by the transferred T cells.

E2 Therapy Decreased Expression of Cytokines, Chemokines, and Chemokine Receptors

To gain insights about the cellular and molecular mechanisms of E2 responses in the spinal cord, we evaluated relative expression levels of proinflammatory cytokines (IFN-γ, TNF-α, IL-1β) and various chemokines (RANTES, MIP-2, IP-10) and chemokine receptors (CCR1, CCR2, CCR6, CCR7, CCR8) known to be involved in inflammatory tissue reactions (Table 3). Comparisons between untreated and E2-treated spinal cords were performed (Table 3) and revealed striking differences in the effect of

Table 3. Gene Expression Was Quantified by Real-Time PCR from Spinal Cord cDNA of E2-Treated Mice without Signs of Disease and Sick Mice with Moderate-Severe or Severe Clinical Signs of EAE

GENE	Fold change		
	Esr1+/+ T-cells → *Esr1*+/+ WT	*Esr1*+/+ T-cells → *Esr1*+/+ SCID	*Esr1*$^{-/-}$ T-cells → *Esr1*+/+ SCID
IFN-γ	−42**	−246*	−90**
TNF-α	−58*	−12*	−267*
IL-1β	−13*	−17**	−11**
RANTES	−108*	−121**	−30*
MIP-2	−166*	−86*	−302*
IP-10	−13***	−30*	−147**
CCR1	−83	−77	−83*
CCR2	−8*	−11*	−134*
CCR6	−108*	−10*	−60*
CCR7	−293*	−237*	−82*
CCR8	−297*	−134*	−82*

For all genes, relative expression level was determined by normalization to L32. Fold change (FC) in mRNA expression was calculated as x/y, where x = relative expression units detected in untreated recipient, y = relative expression units detected in E2-treated recipient. Decreased expression because of E2 treatment was assigned a negative value. Both x and y values were equal to the mean from two independent experiments. *P* values were determined using Student's *t*-test and only $P \leq 0.05$ was considered significant.

*, $P = 0.05–0.01$, **, $P = 0.0001$, ***, $P \leq 0.0001$.

E2 that depended on the presence of recipient-derived nonspecific T cells (comparing WT versus SCID recipients of *Esr1*+/+ T cells) or that depended on T-cell expression of *Esr1* (comparing E2 responses in *Esr1*+/+ SCID mice with disease induced by *Esr1*+/+ versus *Esr1*−/− T cells). E2-induced changes in gene expression in the spinal cord are expressed as the fold change of gene expression in E2-treated mice compared to untreated mice (Table 3). The comparison between *Esr1*+/+ T cells → *Esr1*+/+ WT recipients and *Esr1*+/+ T cells → *Esr1*+/+ SCID recipients revealed enhanced E2-induced down-regulation of IFN-γ expression in the absence of recipient T cells in *Esr1*+/+ SCID mice (Table 3, center column, −246) compared to *Esr1*+/+ WT mice (Table 3, left column, −42) suggesting that E2 down-regulates, either directly or indirectly IFN-γ expression by transferred encephalitogenic T cells. Enhanced down-regulation of TNF-α (−58 versus −12), MIP-2 (−166 versus −86) and CCR8 (−297 versus −134) in the presence of recipient-derived lymphocytes in *Esr1*+/+ recipients suggests that E2 down-regulates these mRNAs primarily on recipient-derived cells or cells recruited nonspecifically to the CNS. Other E2 effects on cytokines and receptor expression did not vary significantly depending on the presence or absence of recipient-derived lymphocytes when comparing *Esr1*+/+ WT versus *Esr1*+/+ SCID recipients.

A comparison between *Esr1*+/+ T cells → *Esr1*+/+ SCID recipients and *Esr1*−/− T cells → *Esr1*+/+ SCID recipients revealed enhanced E2-induced down-regulation of TNF-α, MIP-2, IP-10, CCR2, and CCR6 in mice lacking *Esr1* expression on T cells compared to mice with encephalitogenic *Esr1*+/+ T cells, and enhanced down-regulation of IFN-γ, RANTES, and CCR7 in mice with *Esr1*+/+ encephalitogenic T cells (Table 3). Therefore, the E2-induced gene expression changes in mice possessing *Esr1*+/+ T cells (*Esr1*+/+ T cells → *Esr1*+/+ WT and *Esr1*+/+ T cells → *Esr1*+/+ SCID) may include direct effects of E2 on T cell gene expression (eg, down-regulation of expressed proinflammatory genes in the CNS) and these direct effects on T-cell gene expression are not essential for E2-mediated protection. In contrast, E2-induced gene expression changes by nonlymphoid cells in SCID recipients of *Esr1*−/− T cells (eg, decreased TNF-α, MIP-2, IP-10, CCR2, and CCR6 mRNA expression) may result from direct effects of E2 on nonlymphoid cells whose E2 response is essential for protection.

Discussion

Previous reports showed effective treatment of clinical EAE with 17β-estradiol (E2).[27] The protective effect of E2 was associated with diminished migration of encephalitogenic T cells into the CNS and effects on dendritic cells and macrophages.[28,29] Recently, we also showed using knockout mice that *Esr1* is required for the protective effect of E2 on EAE. The pathogenic role for myelin-specific T lymphocytes in EAE suggested that E2-mediated protection from EAE might be mediated through direct effects on *Esr1*-positive T lymphocytes. We examined this possibility by evaluating E2-protective effects in mice with EAE induced after adoptive transfer of *Esr1*+/+ versus *Esr1*−/− encephalitogenic T cells.

E2 treatment protected mice from EAE and did not depend on *Esr1* expression by encephalitogenic myelin-specific T cells. This demonstrated that the disease-initiating lymphocytes are not the primary E2-responsive cells and implicates other cell types as the primary E2-responsive cells in EAE. The clinical response to E2 was associated with decreased mononuclear cells (total cells, $CD4^+$ and $CD8^+$ lymphocytes, and $CD11b^+$ macrophages and dendritic cells) in the CNS. The numbers of T cells expressing inflammatory adhesion markers VLA-4 and LFA-1 in the brain was reduced in E2-protected versus sick mice (Figure 2), indicating that E2 treatment blocks entry of T cells into the CNS indirectly and independent of E2 effects on adhesion molecule expression by encephalitogenic T cells. An absence of inflammation

and demyelination, pathological disease in all E2-protected mice regardless of *Esr1* expression by encephalitogenic T cells further suggests that encephalitogenic T cells do not directly mediate the E2 treatment response and nonlymphoid cells are the primary E2-responsive cells.

The clinical response to E2 was also associated with a dramatic decrease in absolute numbers of $CD11b^+$, myelomonocytic lineage-derived macrophages, and dendritic cells in the CNS. $CD11c^+$ $CD11b^+$ dendritic cells and $CD45^{high}$ $CD11b^+$ macrophages decreased in absolute numbers and relative abundance in the CNS independent of *Esr1* expression by T cells. $CD45^{intr}$ $CD11b^+$ microglia remained relatively stable in absolute terms but increased in relative abundance because of the E2-induced drop in total cells within the CNS. These results suggest that nonlymphoid *Esr1*+/+ cells such as macrophages and dendritic cells, rather than T cells, are the primary E2-responsive cells.

Certain E2-induced gene expression changes detected by quantitative reverse transcriptase-polymerase chain reaction demonstrated that T cells expressing *Esr1* appeared to respond directly to E2 in these experiments. Thus, E2 levels achieved with the duration, route, and dose used here were effective at inducing immunologically relevant responses by *Esr1*+/+ T cells. However, such direct E2 responses by T cells were not required to induce protection in response to E2 because *Esr1*−/− T cells also supported the E2 protective response in T-cell-deficient SCID mice. Other cell types, besides encephalitogenic T cells or other T cells are likely to be directly responsive to E2 and are therefore the primary cellular element for mediating protection. In contrast to T cells, nonlymphoid *Esr1*+/+ cells were required for down-regulated expression of TNF-α, MIP-2, IP-10, and CCR2 in response to E2 and this was independent of *Esr1* expression by T cells. Thus, these E2-induced gene expression changes depended on nonlymphoid cells independent of *Esr1* expression by T cells and are therefore implicated as crucial participants in the molecular mechanism of E2 protection.

Among the down-regulated expressed genes, down-regulation of TNF-α and MIP-2 appeared to be essential for E2-induced protection in EAE because substantial down-regulation of these gene products was uniformly observed in E2-protected mice. E2-induced down-regulation of TNF-α expression may prevent secondary induction of MIP-2 expression by TNF-α-responsive macrophages or astrocytes.[30–32] Decreased MIP-2 expression would be expected to cause diminished recruitment of CCR2-expressing myelomonocytic lineage cells (blood monocytes and tissue macrophages) from the circulation.[33–36] Therefore, our detection of reduced CCR2 mRNA expression in the spinal cord was likely because of the diminished recruitment of CCR2-expressing monocytes/macrophages because of decreased MIP-2. Although beyond the scope of the present study, future experiments evaluating the phenotype and expressed genes of blood cells before CNS infiltration should contribute additional useful information necessary for understanding the development of the inflammatory response during disease and E2 protection.

E2-induced down-regulation of certain gene products (IP-10, CCR2, and CCR6) appeared to occur to the greatest degree in SCID mice with EAE induced by *Esr1*−/− T cells and to a lesser degree in mice possessing T cells with *Esr1* (eg, SCID mice with *Esr1*+/+ encephalitogenic T cells or immunocompetent WT mice). Because E2-induced down-regulation of these genes was mediated primarily through E2-responsive *Esr1*+/+ nonlymphoid cells, we expected to detect this primary E2 response even when E2-responsive T cells were present. Our inability to detect down-regulation of IP-10, CCR2, and CCR6 in mice with E2-responsive T cells to the same degree as in the absence of E2-responsive T cells may be related to differences between E2 responses expressed by lymphoid versus nonlymphoid cells. As an example of this, CCR8 was down-regulated to the greatest extent by E2 only in mice possessing *Esr1*+/+ T lymphocytes, even though all mice used in these studies possessed E2-responsive nonlymphoid cells. These observations suggest that changes in IP-10, CCR2, and CCR6 expression by nonlymphoid cells may be crucial for E2-induced protection despite an inability to detect such expression changes in the presence of E2-responsive lymphocytes to the same degree as in mice without E2-responsive lymphocytes.

In summary, 17β-estradiol protected mice from EAE induced by adoptively transferred myelin-specific T lymphocytes. Protection was observed as a virtually complete absence of clinical neurological deficits and an absence of inflammatory and other pathological changes in the CNS and did not depend on E2-reponsive lymphocytes. $CD4^+$ and $CD8^+$ lymphocytes, monocytes/macrophages, and $CD11c^+$ dendritic cells were each present in greatly reduced numbers in the CNS of E2-protected mice compared to unprotected, untreated mice. Microglial cell numbers remained relatively stable with E2 treatment. E2-induced protection was mediated through nonlymphoid E2-responsive cells. E2-induced down-regulation of TNF-α and MIP-2 at the transcriptional level was consistently observed, suggesting a primary role for TNF-α-producing macrophages and dendritic cells in E2 protection.[28] A sequential regulatory cascade in which TNF-α induces MIP-2 expression in the CNS may be primarily responsible for recruiting $CCR2^+$ MIP-2-responsive monocyte/macrophage cells to the diseased CNS in the absence of E2. E2 induced a reduction in TNF-α, MIP-2, and CCR2 expression; prevented CNS inflammation; and protected mice from developing disease. Our results provide the first demonstration that the protective effect of E2 in EAE is mediated by nonlymphoid cells capable of modulating this T-cell-mediated autoimmune response. This raises the possibility that nonlymphoid cells such as bone marrow-derived dendritic cells and macrophages are the primary E2-responsive cells in EAE. Future experiments using *Esr1*+/+ and *Esr1*−/− bone marrow chimeric mice with EAE induced by *Esr1*−/− T cells will be conducted to identify the developmental origins of the primary E2-responsive cells.

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