The Protective Effect of 17β -Estradiol on Experimental Autoimmune Encephalomyelitis Is Mediated through Estrogen Receptor- α

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Low-dose estrogen (E2) treatment significantly inhibits the clinical signs and histopathological lesions of experimental autoimmune encephalomyelitis (EAE), and is being used in clinical trials to treat multiple sclerosis. To assess the role of intracytoplasmic estrogen receptors in mediating suppression of EAE, we studied mice with disrupted estrogen receptor- α (*Esr1*) and - β (*Esr2*) genes. We demonstrate that the protective effect of E2 is abrogated in B6.129-*Esr1*^{tm1Unc} mice (*Esr1*^{-/-}) but not in B6.129-*Esr2*^{tm1Unc} mice (*Esr2*^{-/-}). The loss of E2-mediated protection from EAE in *Esr1*^{-/-} mice immunized with the encephalitogenic MOG-35–55 peptide was manifested phenotypically by the development of severe acute clinical signs and histopathological lesions even in the presence of moderately high serum E2 levels. This is in contrast to C57BL/6 wild-type (WT) mice and *Esr2*^{-/-} mice in which E2 treatment resulted in comparable serum levels and markedly suppressed clinical signs of EAE and abolished inflammatory lesions in the CNS. This pattern showing a lack of E2-dependent inhibition of EAE in *Esr1*^{-/-} mice was mirrored by an enhanced rather than a reduced secretion of TNF- α , IFN- γ , and interleukin (IL)-6 in MOG-specific splenocytes and a lack of inhibition of message for inflammatory cytokines, chemokines and chemokine receptors in CNS tissue. These results

indicate that the immunomodulatory effects of E2 in EAE are dependent on *Esr1* and not *Esr2* signaling. (Am J Pathol 2003, 163:1599–1605)

Autoimmune diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), Grave's disease, systemic lupus erythematosus (SLE), and myasthenia gravis, are sexually dimorphic in that they occur more frequently in women than in men.¹ This dimorphism may be due to multiple factors, but gender-related differences in immune responsiveness and sex hormones are likely to play a significant role.² It is well documented that the first clinical symptoms of MS develop post-puberty.³ Additionally, increased levels of sex hormones produced during pregnancy are associated with a significant reduction in the severity of MS, while clinical symptoms are often exacerbated postpartum, a time characterized by significant alternations in sex hormone levels.^{3–5}

We demonstrated previously that administration of low doses of 17β -estradiol reduced the severity of experimental autoimmune encephalomyelitis (EAE) in several different inbred strains of female mice.^{6–8} The exact E2-sensitive immune-related target genes have not been fully characterized. Our data from DNA microarray studies in BV8S2 transgenic mice suggests that E2 down-regulates several members of the TNF gene family including TNF- α and Pgrp, and the chemokine RANTES, and up-regulates the expression of several cytokines, chemokines/receptors, adhesion molecules, and activation markers.⁹ The effects of E2 on gene expression in pathogenic, recruited, and regulatory cells in EAE are likely to be the result of estrogen receptor-mediated signaling.

Although E2 can have a protective effect on susceptibility to EAE, much remains to be learned about the

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molecular mechanisms underlying its immunomodulatory activity and regulation of the sexual dimorphism and pathogenesis of autoimmune diseases.^{6–11} A major question is the nature of the signaling pathways underlying these effects. There is considerable controversy as to whether or not E2 exerts its biological effects directly or indirectly on immune cells and whether or not they are the result of genomic responses. To elicit a classical genomic response, and to be a direct target for E2, the cells of the immune system must express the cognate receptors. The first E2 receptor to be cloned was the E2 receptor- α gene (*Esr1*).¹² Among cells relevant to the functioning of the immune system, *Esr1* is expressed by thymocytes,^{5,6} thymic epithelial cells,⁵ T cells,^{7,8} B lymphocytes and their precursors,^{9,10} and non-hematopoietic bone marrow cells.^{9,11} The existence of a second iER, estrogen receptor- β (*Esr2*), was identified¹² and has been shown to be expressed by non-hematopoietic bone marrow cells¹³ and in the human thymus and spleen.^{14,15}

The precise roles of these two receptors in regulating cell-specific responses are still not well defined. However, it is becoming evident that *Esr1* and *Esr2* may be responsible for regulating different biological functions based on their expression patterns, localization profiles, and protein structures.¹⁴ For example, *Esr1* and *Esr2* differ in their C-terminal ligand-binding domains and in their N-terminal transactivator domains. In addition to these two cytoplasmic receptors, there is also a membrane-associated E2 receptor (mER) that may play a role in mediating the non-genomic actions of E2.¹⁵ However, mER regulation of gene expression cannot be fully excluded since some phenotypes elicited by steroid hormones appear to be the result of membrane receptor activation of G-protein-coupled second messenger pathways.¹⁶ In the current study, we use B6.129-*ESR1* mice lacking *Esr1* and B6.129-*Esr2*^{tm1Unc} mice lacking *Esr2* to address for the first time which ER mediates the protective effect of E2 on EAE.

Materials and Methods

Animals

Female B6.129-*Esr1*^{tm1Unc} (*Esr1*–/–), B6.129-*Esr2*^{tm1Unc} (*Esr2*–/–), and C57BL/6 wild-type control littermate mice were purchased from Taconic Laboratory (Germantown, NY). The mice were housed and cared for in the Animal Resource Facility at the Portland VA Medical Center according to institutional guidelines.

Hormone Treatment

For treatment of mice with 17 β -estradiol, 3-mm pellets containing 2.5 mg of E2 (Innovative Research of America, Sarasota, FL), providing a constant continuous controlled release of hormone over a period of 60 days, were implanted subcutaneously on the dorsal back of each mouse 7 days before immunization for induction of EAE. Control animals were sham-operated and implanted with pellets containing saline.

Induction of Active EAE

Mice were immunized with 400 μ g of MOG-35–55 peptide in complete Freund's adjuvant (CFA) containing 200 μ g of *Mycobacterium tuberculosis* H37Ra by subcutaneous injection over four sites on the flank, with additional injections of Pertussis toxin (Ptx) on days 0 and 2.⁷ Mice were examined daily for clinical signs of EAE according to the following scale: 0, no signs; 1, limp tail; 1.5, moderate hind limb weakness with difficulty in righting; 2, moderate hind limb weakness without ability to right itself; 2.5, moderate hind limb weakness (waddling gait) without ability to right itself; 3, moderately severe hind limb weakness with the ability to walk upright for only a few steps; 3.5, moderately severe hind limb weakness with paralysis of one limb; 4, severe hind limb weakness; 4.5, severe hind limb weakness with mild forelimb weakness; 5, paraplegia with no more than moderate forelimb weakness; 5.5, paraplegia with severe forelimb weakness (quadriplegia); and 6, moribund condition.

IL-6 Quantification

Splenic cells were cultured at 4×10^6 /ml and stimulated with the appropriate Ag in 24-well culture plates. Cell culture supernatants were recovered after 72 hours. Measurement of cytokines was performed by ELISA using cytokine-specific capture and detection Abs (BD Pharmingen, San Diego, CA). A standard curve for IL-6 was generated using recombinant mouse cytokines (BD Pharmingen), and the concentration of cytokine in the cell supernatants was determined by interpolation from the appropriate standard curve.

Cytometric Bead Array (CBA)

TNF- α , IFN- γ , IL-5, IL-4, and IL-2 were detected using the mouse inflammation cytokine CBA kit from BD Biosciences (San Jose, CA). Fifty μ l of cell culture supernatant was mixed with 50 μ l of the mixed capture beads and 50 μ l of the mouse phycoerythrin detection reagent. The tubes were incubated at room temperature for 2 hours in the dark, followed by a wash step. The samples were then resuspended in 400 μ l of wash buffer before acquisition on the FACScan. The data were analyzed using CBA software. Standard curves were generated for each cytokine using the mixed bead standard provided in the kit, and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve.

RNase Protection Assay (RPA)

Total RNA was extracted from spinal cords (SC) using the STAT-60 reagent (Tel-Test, Friendswood, TX). Chemokine expression was determined using the RiboQuant RPA kit to assess the expression of chemokines and chemokines receptors (PharMingen) according to manufacturer's instructions.⁶

Table 1. Susceptibility to MOG 35–55 Induced EAE in Placebo- and E₂-Treated C57BL/6, B6.129-*Esr1*^{-/-}, and B6.129-*Esr2*^{-/-} Mice

Treatment	Incidence	Onset	Peak	CDI	E ₂ (pg/ml)	T4 (pg/ml)	Thymic Index (mg/g)	Uterine Index (mg/g)
C57BL/6								
Placebo	10/10	9.9 ± 1.8	4.8 ± 1.0	61.3 ± 20.8	20 ± 18	36.2 ± 34.6	3.7 ± 0.4	4.5 ± 1.7
E ₂	3/10 ^{††}	21.2 ± 3.8 ^{*††§¶}	0.6 ± 1.3 ^{*††§}	1.1 ± 2.5 ^{*††§}	1,795 ± 601.3 ^{*†§}	131.2 ± 120.5 ^{††}	1.8 ± 0.2 ^{*††§¶}	13.3 ± 6.1 ^{*††§}
B6.129- <i>Esr1</i> ^{-/-}								
Placebo	8/8	12.4 ± 2.7	4.6 ± 0.8	58.5 ± 14.7	12 ± 20.5 [†]	2595.6 ± 200.8 ^{*§¶}	2.4 ± 0.5	6.8 ± 0.7
E ₂	8/8	16.1 ± 2.7 ^{* †§¶}	5.3 ± 0.7 ^{†¶}	60.2 ± 2.7 ^{†¶}	1,432 ± 238 ^{*†§}	1635.6 ± 1306.9 ^{*§¶}	1.3 ± 0.2 ^{* ††§}	4.9 ± 0.9
B6.129- <i>Esr2</i> ^{-/-}								
Placebo	5/5	12.0 ± 1.0	5.5 ± 0.0	77.1 ± 9.2	36.8 ± 10.3	29.6 ± 18.4	2.8 ± 0.9	4.8 ± 2.8
E ₂	1/4 [†]	30.0	0.5 ± 1.0 ^{*††§}	0.5 ± 1.0 ^{*††§}	1171.5 ± 378.8 ^{*†§}	75.0 ± 32.4 ^{*†}	1.3 ± 0.3 ^{* †§}	12.7 ± 2.6 ^{*††§}

*P value ≤ 0.05, as compared to C57BL/6 treated with placebo pellet.

†P value ≤ 0.05, as compared to B6.129-*Esr1*^{-/-} treated with placebo pellet.

††P value ≤ 0.05, as compared to B6.129-*Esr1*^{-/-} treated with E₂ pellet.

§P value ≤ 0.05, as compared to B6.129-*Esr2*^{-/-} treated with placebo pellet.

¶P value ≤ 0.05, as compared to B6.129-*Esr2*^{-/-} treated with E₂ pellet.

||P value ≤ 0.05, as compared to C57BL/6 treated with E₂ pellet.

Onset, peak, and CDI are presented as the mean trait value ± SD. The CDI was determined by summing the daily clinical scores.

Histology

Representative mice were selected from the E₂ and placebo-treated control group. Spinal cords were isolated and fixed in 10% paraformaldehyde. Transverse sections of the spinal cords were stained with Luxol Fast Blue-Periodic acid or with hematoxylin and eosin (H&E) and analyzed by light microscopy.

E₂ and Testosterone Serum Levels

Serum E₂ and testosterone levels were measured using a radioimmunoassay as described previously.⁶

Statistical Analysis

Differences in the incidence of EAE in the respective mouse strains was tested using χ^2 . A one-way analysis of variance and Student-Newman-Keuls multiple comparisons test was used to evaluate significant differences in quantitative EAE trait values (day of onset, E₂ levels, T4 levels, thymic index and uterine index). The Kruskal-Wallis test was used to evaluate non-parametric EAE trait values (peak score and CDI score). In all tests, *P* values ≤ 0.05 were used as the significance threshold.

Results

EAE in *Esr1*^{-/-} and *Esr2*^{-/-} Mice

We sought to determine whether there were differences between *Esr1* and *Esr2* in mediating the inhibitory effects of E₂ on EAE. Thus, B6.129-*Esr1*^{tm1Unc} (*Esr1*^{-/-}) mice, B6.129-*Esr2*^{tm1Unc} (*Esr2*^{-/-}) mice, and their C57BL/6 WT littermate controls were immunized with MOG-35–55 peptide + CFA + Ptx in the presence of implanted E₂ or placebo pellets, and monitored for clinical and histological signs EAE (Table 1 and Figures 1 and 2). All of the WT C57BL/6 mice treated with placebo pellets developed severe EAE, and as expected from our previous studies,^{6–9} treatment of the WT mice with E₂ pellets mark-

edly reduced the incidence and severity of clinical and histological disease. Interestingly, both *Esr1*^{-/-} and *Esr2*^{-/-} mice treated with placebo pellets developed EAE that was nearly indistinguishable from WT controls. Of critical importance, however, treatment with E₂ pellets had no inhibitory effect on the severity of clinical or histological disease in *Esr1*^{-/-} mice, although onset of EAE was delayed by 4 days. In contrast, the identical treatment with E₂ almost completely inhibited clinical and histological EAE in *Esr2*^{-/-} mice (Table 1 and Figure 1).

We also evaluated the lack of *Esr1* and *Esr2* on other E₂ functions in placebo- or E₂-treated mice with EAE. Quantification of serum E₂ levels revealed a low but measurable concentration of endogenous E₂ in *Esr1*^{-/-} placebo-treated mice that was similar to that present in C57BL/6 WT littermate controls, but lower than in *Esr2*^{-/-} mice (Table 1). Moreover, treatment with E₂ pellets produced comparable levels of serum E₂ in all three mouse strains, indicating that the lack of functional estrogen receptors did not effect E₂ degradation. As

Figure 1. Wild-type C57BL/6, *Esr1*^{-/-}, and *Esr2*^{-/-} female mice were implanted with 2.5 mg 17 β -estradiol (E₂) or placebo pellets, respectively, 1 week before the induction of EAE. E₂ treatment suppressed actively induced EAE in C57BL/6 and *Esr2*^{-/-} mice, while in *Esr1*^{-/-} mice this protective effect was lost. The data show mean clinical EAE scores for each day for C57BL/6 female mice treated with saline or E₂ pellets, B6.129-*Esr1*^{-/-} females treated with saline or E₂ pellets, and B6.129-*Esr2*^{-/-} females treated with saline or E₂ pellets.

Figure 3. Cytokine expression in spleens of placebo-treated and E2-treated C57BL/6, *Esr1*^{-/-}, and *Esr2*^{-/-} mice. Spleens were recovered from immunized mice at the peak of disease (15 days post-immunization). A total of 4×10^6 cells were stimulated with 10 μ g MOG-35–55 peptide for 72 hours before removal of the supernatants for assessment of cytokine secretion by CBA for TNF- α , IFN- γ , IL-2, IL-4, and IL-5, or by ELISA for IL-6.

expected, *Esr1*^{-/-} mice had no significant increase in uterine index [expressed as uterine weight (mg) divided by body weight (g)] compared to WT controls and *Esr2*^{-/-} mice. However, E2 treatment significantly increased the uterine index nearly three-fold in WT and *Esr2*^{-/-} mice but not *Esr1*^{-/-} mice. In contrast, lack of either *Esr1* or *Esr2* resulted in a moderate decrease in thymic index compared to WT controls, and treatment with E2 strongly diminished thymus weight in all three mouse strains. Testosterone levels were low but detectable in WT and *Esr2*^{-/-} mice, but were markedly increased in *Esr1*^{-/-} mice, consistent with a previous report.¹⁷ Taken together, these results are consistent with previous findings regarding the role of *Esr1* and *Esr2* signaling, and serve as internal controls for the observed effects on EAE.^{18–22}

Cytokine, Chemokine, and Chemokine Receptor Production by *Esr1*^{-/-} and *Esr2*^{-/-} Mice

In accordance with our previous studies,^{6–9} we observed that E2 treatment significantly reduced the secretion of TNF- α and IFN- γ by MOG-35–55 peptide-stimulated spleen cells recovered from WT mice at the peak of EAE (Figure 3). A similar result occurred in *Esr2*^{-/-} mice, although levels of both cytokines were reduced compared to WT placebo-treated mice. In contrast, E2 treatment significantly enhanced secretion of TNF- α and IFN- γ in *Esr1*^{-/-} mice with EAE compared to treatment with placebo (Figure 3). IL-2, IL-4, and IL-5 levels were very low and were not affected by E2 treatment in any mice. Since E2 has been purported to up-regulate serum IL-6 levels through *Esr1* and not *Esr2*,²³ and elevated IL-6 levels have been implicated in mediating suppression of delayed-type hypersensitivity responses in post-burn pa-

Figure 4. RPA analysis of (A) cytokine, (B) chemokine, and (C) chemokine receptor mRNA in spinal cords of protected (E2-treated) and non-protected C57BL/6, *Esr1*^{-/-}, and *Esr2*^{-/-} mice, respectively. Expression of LTn, TNF- α , IFN- γ , RANTES, MIP-1 α , MIP-2, IP-10, and MCP-1, and CCR1, CCR2, and CCR5 was strongly upregulated in spinal cords of placebo-treated C57BL/6, *Esr1*^{-/-}, and *Esr2*^{-/-} mice, as well as E2-treated *Esr1*^{-/-} mice at the peak of EAE. In contrast, E2 treatment greatly reduced expression of all chemokines and chemokine receptors in WT and *Esr2*^{-/-} mice protected from EAE. Data presented are from one representative experiment of two or three total RPA evaluations that showed similar results. $P \leq 0.05$, $** P \leq 0.001$, $*** P \leq 0.0001$ as compared to WT mice treated with the saline pellet.

tients and experimental animals,²⁴ we compared the levels of IL-6 secretion by splenic cells in E2- versus placebo-treated *Esr1*^{-/-} and *Esr2*^{-/-} mice. As is shown in Figure 3, IL-6 production was significantly reduced in E2-treated WT mice without EAE, but was significantly increased in E2-treated versus placebo-treated *Esr1*^{-/-} mice with EAE. These data indicate that the up-regulation of IL-6 is not restricted to *Esr1* signaling and that increased levels of IL-6 do not account for suppression of clinical and histological EAE.

We previously demonstrated that E2 treatment influenced the expression of cytokine, chemokine and chemokine receptor mRNA in the spinal cords of C57BL/6

Figure 2. Histopathological examination of spinal cord sections from C57BL/6, *Esr1*^{-/-}, and *Esr2*^{-/-} mice reveals the lack of protection against EAE in *Esr1*^{-/-} mice treated with 2.5-mg E2 pellet. **A:** C57BL/6 mice treated with placebo pellet with EAE (magnification, $\times 50$). **B:** C57BL/6 mice treated with 2.5-mg E2 pellet, fully protected from EAE. No visible signs of inflammation were present within spinal cord tissue. **C:** B6.129-*Esr1*^{-/-} mice treated with placebo pellet with EAE. **D:** B6.129-*Esr1*^{-/-} mice treated with 2.5-mg E2 pellet with EAE. Several foci of inflammation are clearly evident within the spinal cord tissue. **E:** B6.129-*Esr2*^{-/-} mice treated with placebo pellet with EAE. **F:** B6.129-*Esr2*^{-/-} mice treated with 2.5-mg E2 pellet, fully protected from EAE. No visible signs of inflammation were present within spinal cord tissue. Histopathological sections for placebo- and E2-treated *Esr2*^{-/-} mice were essentially the same as in C57BL/6 mice.

mice with EAE.^{8–9} mRNA levels for many of the cytokines (Lymphotoxin, TNF- α , and IFN- γ), chemokines (RANTES, MIP-1 α , MIP-2, IP-10, and MCP-1) and chemokine receptors (CCR1, CCR2, and CCR5) were detectable at similar levels in the spinal cords of placebo-treated WT, *Esr1*^{–/–}, and *Esr2*^{–/–} mice with clinical signs of EAE (Figure 4). Treatment of EAE with E2 significantly reduced the expression of all detected cytokines, chemokines, and chemokine receptors in WT C57BL/6 mice and *Esr2*^{–/–} mice, but did not significantly change the level or pattern of expression in CNS tissue from E2-treated *Esr1*^{–/–} mice that developed EAE (Figures 4, A to C). Thus, the inhibitory effect of E2 on inflammatory factors in CNS that was highly significant in C57BL/6 and *Esr2*^{–/–} mice was lost in *Esr1*^{–/–} mice.

Discussion

We previously demonstrated that low-dose E2 therapy is beneficial in the treatment of EAE in two different inbred mouse strains.^{6–9} We now demonstrate for the first time that the protective effect of E2 is abrogated in *Esr1*^{–/–} mice, but not in *Esr2*^{–/–} mice. The loss of the protective effect of E2 in *Esr1*^{–/–} mice immunized with the encephalitogenic MOG-35–55 peptide was manifested by the development of severe clinical and histological EAE even in the presence of moderately high serum E2 levels. This is in contrast to C57BL/6 WT mice and *Esr2*^{–/–} mice, in which E2 treatment resulted in comparable serum E2 levels and markedly suppressed clinical signs of EAE and abolished inflammatory lesions in the CNS. This pattern showing a lack of E2-dependent inhibition of EAE in *Esr1*^{–/–} mice was mirrored by an enhanced rather than a reduced secretion of TNF- α , IFN- γ , and IL-6 in MOG-specific splenocytes and a lack of inhibition of message for inflammatory cytokines, chemokines, and chemokine receptors in CNS tissue.

Although the severity of clinical and histological EAE was not diminished in E2-treated *Esr1*^{–/–} mice, these mice did exhibit a 4-day delay in disease onset compared to C57BL/6 controls or *Esr2*^{–/–} mice. This may be the result of residual *Esr2* signaling,²⁵ which leads to less efficient E2 protection. MOG-specific splenocytes from placebo-treated *Esr1*^{–/–} and *Esr2*^{–/–} mice had strongly reduced levels of secreted TNF- α and IFN- γ compared to WT controls, suggesting that lack of *Esr1* or *Esr2* might limit production of these cytokines. However, treatment with E2 strongly increased the secretion of these cytokines to WT levels in *Esr1*^{–/–} mice, suggesting a possible enhancing effect of E2 mediated through the residual *Esr2* gene. E2 treatment also significantly enhanced secretion of IL-6 in MOG peptide-stimulated splenocytes from *Esr1*^{–/–} mice, indicating that E2 up-regulation of IL-6 is not restricted to *Esr1* signaling and that increased levels of IL-6 do not account for suppression of EAE, as proposed previously.^{23,24} In addition, E2 treatment of *Esr1*^{–/–} mice did not change the expression profile in the spinal cord of the cytokines lymphotoxin, TNF- α , and IFN- γ ; the chemokines RANTES, MIP-1 α , MIP-2, IP-10, and MCP-1; and the chemokine

receptors CCR1, CCR2, and CCR5, in contrast to C57BL/6 WT and *Esr2*^{–/–} mice in which E2 treatment reduced the levels of all of these inflammatory factors. In this regard, it is pertinent that Wang et al²⁶ reported that the two-ligand/receptor pairs, CCL5/CCR5 and CXCL10/CXCR3, may play a role during active MS and serve as predictors of future disease activity. Also Eikelenboom et al²⁷ showed an increase in CCR5⁺ and CXCR3⁺ T cells in both the blood and cerebrospinal fluid of MS patients.

Based on incomplete development of the mouse reproductive tract (ovaries) in B6.129-*Esr1*^{–/–} mice, it has been suggested that the action of *Esr2* is directly dependent on the presence of a functional *Esr1* gene.²⁸ On the other hand, E2 continues to provide protection against vascular injury in mice in which either *Esr1* or *Esr2* has been disrupted, suggesting that each of the two known E2 receptors is sufficient to mediate vascular protection.²⁹ In ischemic injury within the brain, E2 can use either ER subtype,³⁰ or alternatively, protection may be receptor-independent.³¹ Similarly, in our study, lack of *Esr1* or *Esr2* resulted in a moderate decrease in thymus weight compared to WT controls, and treatment with E2 strongly diminished thymus weight in all three mouse strains. E2 and estrogen receptor are known to effect thymic development and function,^{20–22} and our results support a role for either *Esr1* or *Esr2*. The spectrum of biological activities mediated by E2 receptor subtypes in diverse disease settings may be explained by: 1) the differences in tissue distribution of the subtypes, 2) the complexity of E2 receptor co-activators which may be different for each cell type, or 3) the existing complexities in the nature of E2 protective mechanisms, which include classical (genomic) and nonclassical (nongenomic) action.³²

Our data strongly implicate the classical (genomic) action of E2 in protection against EAE, because we observed a pronounced E2-dependent regulation of cytokine secretion in spleen and inhibition of message for cytokines, chemokines, and chemokine receptors in the spinal cord of immunized mice. Our study demonstrates for the first time that these E2-regulated immunomodulatory effects are mediated by selective signaling through *Esr1*. Preliminary studies indicate that iERs are present on CD4⁺ T cells and monocytes, and it remains plausible that *Esr1* on immunocytes may directly mediate E2 effects on EAE.

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