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Estrogen Receptor-1 (*Esr1*) and -2 (*Esr2*) Regulate the Severity of Clinical Experimental Allergic Encephalomyelitis in Male Mice

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Estrogens and estrogen-receptor signaling function in establishing and regulating the female immune system and it is becoming increasingly evident that they may play a similar role in males. We report that B10.PL/SnJ male mice with a disrupted estrogen receptor-1 (α) gene (*Esr1*$^{-/-}$) develop less severe clinical experimental allergic encephalomyelitis (EAE) compared to either *Esr1*$^{+/-}$ or wild-type (*Esr1*$^{+/+}$) controls when immunized with myelin basic protein peptide Ac1-11 (MBP_{Ac1-11}). In contrast, the disease course in B10.PL/SnJ male mice with a disrupted estrogen receptor-2 (β) gene (*Esr2*$^{-/-}$) does not differ from that of wild-type (*Esr2*$^{+/+}$) mice. However, *Esr2*$^{+/-}$ mice do develop more severe clinical disease with an earlier onset indicating that heterosis at *Esr2* plays a significant role in regulating EAE in males. No significant differences in central nervous system histopathology or MBP_{Ac1-11}-specific T-cell responses as assessed by proliferation and interleukin-2 production were observed as a function of either *Esr1* or *Esr2* genotype. An analysis of cytokine/chemokine secretion by MBP_{Ac1-11}-specific T cells revealed unique *Esr1* and *Esr2* genotype-dependent regulation. Interferon-γ secretion was found to be negatively regulated by *Esr1* whereas interleukin-6 and tumor necrosis factor-α secretion exhibited classical *Esr2* gene dose responses. Interestingly, MCP-1 displayed distinctively unique patterns of genotype-dependent regulation by *Esr1* and *Esr2*. The contribution of the hematopoietic and nonhematopoietic cellular compartments associated with the heterotic effect at *Esr2* in regulating the severity of clinical EAE was identified using reciprocal hematopoietic radiation bone marrow chimeras generated between male wild-type and *Esr2*$^{+/-}$ mice. Wild-type → *Esr2*$^{+/-}$ mice exhibited EAE equivalent in severity to that seen in *Esr2*$^{+/-}$ → *Esr2*$^{+/-}$ control constructs; both of which were more severe than the clinical signs observed in *Esr2*$^{+/-}$ → wild-type and wild-type → wild-type mice. These results indicate that the heterotic effect at *Esr2* is a function of the nonhematopoietic compartment. ***(Am J Pathol 2004, 164:1915–1924)***

In females both the humoral and cell-mediated immune responses are more active than in males[1] and extensive data indicate that estrogens play a significant role in regulating this immunological sexual dimorphism.[2,3] To be a direct target for estrogens either the cells of the immune system, or the nonhematopoietically derived cellular constituents that support their development and function, must express the appropriate cognate receptors. The first classical intracytoplasmic estrogen receptor (iER) cloned was estrogen receptor-1 (α) (*Esr1*).[4] Among cells relevant to the functioning of the immune system, Esr1 is expressed by thymocytes,[5,6] thymic epithelial cells,[6] T cells,[7,8] B lymphocytes and their precursors,[9,10] and nonhematopoietic bone marrow cells.[9,11] The existence of a second iER, estrogen receptor-2 (β) (*Esr2*), was identified[12] and has been shown to be expressed by nonhematopoietic bone marrow cells[13] and in the human thymus and spleen.[14,15] The precise roles of these two receptors in regulating cell-specific responses are still ill-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.[16,17] In addition to these two cytoplasmic receptors, there may also be a membrane-associated ER

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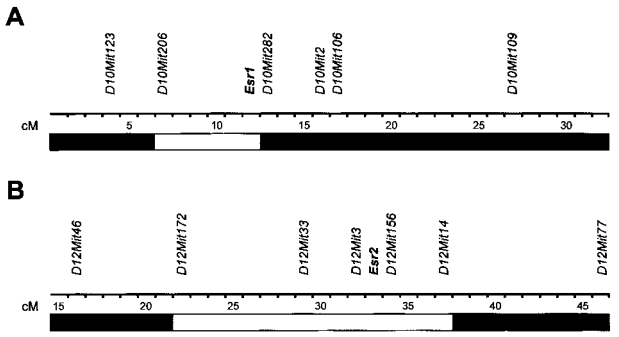

Figure 1. Congenic intervals for B10.PL-*Esr1* (**A**) and B10.PL-*Esr2* (**B**) mice. Boundaries of the congenic intervals for both loci were determined using informative microsatellite markers distinguishing 129-strain alleles from C57BL/6J and C57BL/10J strain alleles. Genotyping was performed as previously described.[26–28] □, 129-strain-derived alleles; ■, C57BL/6J and/or C57BL/10J alleles.

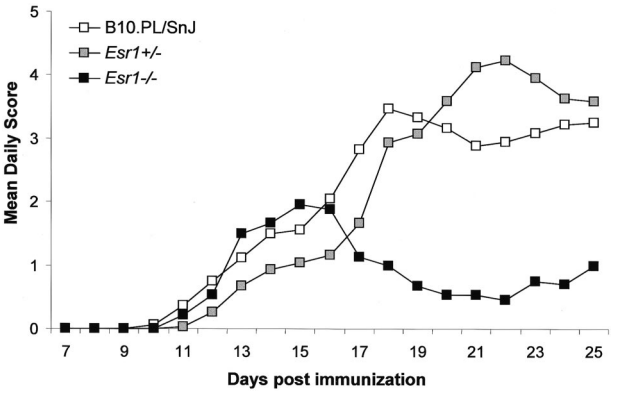

Figure 2. Clinical disease profiles comparing ERKO wild-type B10.PL/SnJ ($n = 18$), B10.PL-*Esr1*$^{+/-}$ ($n = 15$), and B10.PL-*Esr1*^{tm1Unc} ($n = 12$) mice. Male littermates were injected with 400 μg of $MBP_{Ac1\text{-}11}$ + CFA + PTX. Starting on day 7 the animals were monitored daily for clinical signs of EAE and the data are plotted as the mean daily score for all animals studied.

that is believed to play a role in mediating nongenomic responses[18] as well as a number of estrogen-related receptor genes (α, β, γ, and receptor β-like 1; *Esrra*, *Esrrb*, *Esrrg*, and *Esrrbl1*, respectively) that function as ligand-regulated transcription factors that play critical roles in multiple aspects of development, cellular differentiation, and homeostasis.[19]

The effects of estrogens and ER signaling may be prenatal, postnatal, or both[16,17] and their involvement in reproduction, growth, and certain organ systems, including the central nervous system (CNS), is well documented in males.[16,20] However, their role in the male immune system is beginning to be more fully appreciated. Others and we have shown that Esr1 is involved in normal thymic development and T lymphopoiesis[21,22] and in regulating B-cell development[9,10] in male mice. However, the role of iERs in autoimmune disease in males is uncharted. In the current study, we use male B10.PL/SnJ congenic mice possessing either a disrupted estrogen receptor-1 (*Esr1*$^{-/-}$) or estrogen receptor-2 (*Esr2*$^{-/-}$) gene to assess the role of these two iERs in experimental allergic encephalomyelitis (EAE), the principal animal model of multiple sclerosis. In this study we report that the severity of EAE and the secretion of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and MCP-1 by encephalitogenic T cells in male mice exhibit distinctively unique *Esr1* and *Esr2* genotype-dependent regulation.

Materials and Methods

Animals

B10.PL/SnJ mice were purchased from the Jackson Laboratory, Bar Harbor, ME. B10PL.129P2(B6)-*Esr1*^{tm1Unc} (abbreviated B10.PL-*Esr1*^{tm1Unc} or *Esr1*$^{-/-}$) and B10PL.129P2(B6)-*Esr2*^{tm1Unc} (abbreviated B10.PL-*Esr2*^{tm1Unc} or *Esr2*$^{-/-}$) mice were generated by backcrossing B6.129P2-*Esr1*^{tm1Unc} and B6.129P2-*Esr2*^{tm1Unc} mice to B10.PL/SnJ mice for 10 generations.[23,24] This represents a total of a minimum of 20 backcross generations on the C57BL background with respect to 129P2 genomic contamination. Mice were selected at each generation by polymerase chain reaction-based genotyping using primers specific for the disrupted *Esr1* and *Esr2* alleles. The congenic intervals encompassing the two disrupted loci are presented in Figure 1. Animals were maintained in accordance with the Animal Welfare Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals.

Induction of Active EAE

Induction of active disease was as previously described.[25] Briefly, on day 0 mice were immunized with 400 μg of myelin basic protein peptide Ac1-11 ($MBP_{Ac1\text{-}11}$) (Beckman Institute, Palo Alto, CA) emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) by subcutaneous injection over four sites on the flank. On the day of immunization mice received by intraperitoneal injection 75 ng of pertussis toxin (PTX) (List Biological Laboratories Inc., Campbell, CA). Forty-eight hours later each mouse received an additional 200 ng of PTX by intraperitoneal injection.

Evaluation of Clinical and Histopathological EAE

Starting on day 7 after immunization the 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. Clinical disease parameters assessed were incidence and mortality, and the quantitative traits (QTs): day of onset of clinical signs,

Table 1. Susceptibility to MBP_{Ac1-11}-Induced EAE in B10.PL/SnJ, B10.PL-*Esr1*$^{+/-}$, and B10.PL-*Esr1*^{tm1Unc} Male Mice

Genotype	Incidence	Onset	Peak score	SI	CDS	DI	Mortality
B10.PL/SnJ	18/18	15.6 ± 4.8	4.2 ± 1.9	3.2 ± 1.6	35.6 ± 21.6	1.9 ± 1.1	2/18
Esr1$^{+/-}$	15/15	16.5 ± 3.6	5.0 ± 1.3	3.4 ± 1.2	34.9 ± 15.6	1.8 ± 0.8	3/15
Esr1$^{-/-}$	8/12	12.3 ± 1.0	3.3 ± 1.5	1.7 ± 0.8*	14.5 ± 15.8*	0.8 ± 0.8*	0/12
	$\chi^2 = 12.07$	$F = 3.18$	$F = 2.73$	$F = 4.77$	$F = 5.58$	$F = 5.94$	$\chi^2 = 2.70$
P value	0.002	0.05	0.08	0.01	0.007	0.005	0.26

*Significance of differences based on one-way multiple comparisons with Tukey's and Hsu's family error rates at 0.1: *Esr1*$^{-/-}$ < *Esr1*$^{+/-}$ = wild-type.

cumulative disease score (CDS), disease index (DI), peak score, and severity index (SI).[26,27]

Brains and spinal cords (SCs) were dissected from calvaria and vertebral columns, respectively, and fixed by immersion in 10% phosphate-buffered formalin (pH 7.2). After adequate fixation, they were trimmed and representative transverse sections-embedded in paraffin, sectioned at 5 μm, and mounted on glass slides. Sections were stained with hematoxylin and eosin (H&E) for routine evaluation and Luxol fast blue-periodic acid-Schiff reagent for demyelination. Representative areas of the brain and SC, including brain stem, cerebrum, cerebellum, and the cervical, thoracic, and lumbar segments of the SC, were selected for histopathological evaluation based on previous studies.[28] The following components of the lesions were assessed: 1) severity of the lesion as represented by each component of the histopathological assessment; 2) extent and degree of myelin loss and tissue injury (swollen axon sheaths, swollen axons, and reactive gliosis); 3) severity of the acute inflammatory response (predominantly neutrophils); and 4) severity of the chronic inflammatory response (lymphocytes/macrophages). A score was assigned separately to the entire brain and SC for each lesion characteristic based on a subjective scale ranging from 0 to 5. A score of 0 indicates no lesions; 1 indicates minimal; 2, mild; 3, moderate; 4, marked; and 5, severe lesions. Occasional mice had eosinophils admixed with the dominant neutrophilic inflammatory response.

Proliferation Assays

Single cell suspensions of draining lymph node cells were prepared.[29–31] Draining lymph node cells (4×10^5 cells/well) were plated on standard 96-well flat-bottom tissue culture plates for 72 hours at 37°C and 7% CO_2 with and without antigen and in the presence of 0.5 μCi of ^{3}H-thymidine during the last 18 hours. Cells were harvested onto glass fiber filters, and thymidine uptake was determined by liquid scintillation. Data are expressed as corrected mean counts per minute.

Cytokine Assays

Single cell splenocyte suspensions from immunized mice were prepared and suspended at 4×10^6 cells/ml in stimulation media with 20 μg/ml of MBP_{Ac1-11}. Cell culture supernatants were recovered at 72 hours and frozen at −70°C. IL-4 secretion was quantitated by enzyme-linked immunosorbent assay (PharMingen, San Diego, CA) and IFN-γ, TNF-α, IL-2, IL-6, IL-5, IL-10, IL-12, and MCP-1 were simultaneously detected using the mouse inflammation and Th1/Th2 cytokine CBA kits from BD Biosciences (San Jose, CA).[29–31] Fifty μl of sample were mixed with 50 μl of the mixed capture beads and 50 μl of the mouse inflammation phycoerythrin-conjugated detection antibodies. 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 μl of wash buffer before acquisition on the FACScan (BD Bioscience, San Jose, CA). The data were analyzed using the 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. Means and standard deviations were determined using data from individual animals.

Radiation Bone Marrow Chimeras

Radiation bone marrow chimeras were constructed as previously described.[32,33] Briefly, host mice were lethally irradiated and reconstituted by the intravenous injections

Table 2. Comparison of Acute-Early and Chronic-Late Stages of EAE in B10.PL/SnJ, B10.PL-*Esr1*$^{+/-}$, and B10.PL-*Esr1*^{tm1Unc} Male Mice

	Acute-early				Chronic-late			
Genotype	Peak score	SI	CDS	DI	Peak score	SI	CDS	DI
B10.PL/SnJ	4.5 ± 1.6	3.2 ± 0.7	13.7 ± 11.3	1.1 ± 0.9	3.8 ± 2.0	3.3 ± 2.0	21.9 ± 14.8	3.1 ± 2.1
Esr1$^{+/-}$	4.3 ± 1.0	2.5 ± 1.2	8.7 ± 8.8	0.7 ± 0.7	4.7 ± 1.5	3.8 ± 1.7	26.2 ± 12.5	3.7 ± 1.8
Esr1$^{-/-}$	3.3 ± 1.5	2.2 ± 1.1	9.9 ± 10.2	0.8 ± 0.8	2.8 ± 1.3	1.2 ± 0.9*	4.7 ± 6.0*	0.7 ± 0.9*
	$F = 2.07$	$F = 3.30$	$F = 1.07$	$F = 1.01$	$F = 2.97$	$F = 5.80$	$F = 11.20$	$F = 10.93$
P value	0.14	0.05	0.35	0.37	0.06	0.006	<0.001	<0.001

*Significance of differences based on one-way multiple comparisons with Tukey's and Hsu's family error rates at 0.1: *Esr1*$^{-/-}$ < *Esr1*$^{+/-}$ = wild-type.

of 10^7 T-cell-depleted donor bone marrow cells. In this way the following radiation chimeras were generated: wild-type → wild-type, *Esr2*$^{+/-}$ → *Esr2*$^{+/-}$, wild-type → *Esr2*$^{+/-}$ and *Esr2*$^{+/-}$ → wild-type. Chimeras were studied for EAE at 4 to 6 weeks after reconstitution.

Statistical Analysis

Analysis of variance was used to test for significant differences in QT values by genotype for the two lines. Where indicated posthoc one-way multiple comparisons with Tukey's and Hsu's family error rates of 0.1 were used to determine the significance of differences in clinical QT values as a function of genotype. Logistic regression with a Wald chi-square test was used to test for significant qualitative differences by genotype. In all tests, *P* values ≤0.05 were used as the significance threshold.

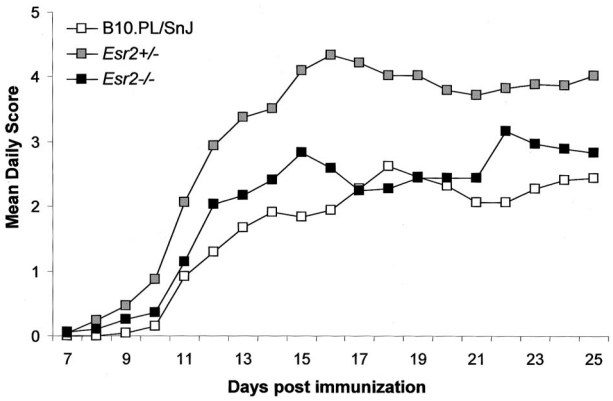

Figure 3. Clinical disease profiles comparing BERKO wild-type B10.PL/SnJ (n = 24), B10.PL-*Esr2*$^{+/-}$ (n = 32), and B10.PL-*Esr2*^{tm1Unc} (n = 18) mice. Male littermates were injected with 400 μg of MBP_{Ac1-11} + CFA + PTX. Starting on day 7 the animals were monitored daily for clinical signs of EAE and the data are plotted as the mean daily score for all animals studied.

Results

*EAE in B10.PL/SnJ, B10.PL-*Esr1$^{+/-}$*, and B10.PL-*Esr1^{tm1Unc} *Male Mice*

To study the role of *Esr1* in regulating susceptibility to EAE in male mice, homozygous wild-type (B10.PL/SnJ), heterozygous B10.PL-*Esr1*$^{+/-}$ (*Esr1*$^{+/-}$), and homozygous *Esr1*-deficient B10.PL-*Esr1*^{tm1Unc} (*Esr1*$^{-/-}$) littermates were immunized with MBP_{Ac1-11} + CFA + PTX, and monitored for clinical EAE (Figure 2). No significant difference in disease onset, peak score, or mortality was detected between wild-type, *Esr1*$^{+/-}$, and *Esr1*$^{-/-}$ mice (Table 1). However, compared to wild-type and *Esr1*$^{+/-}$ mice, *Esr1*$^{-/-}$ males presented with a significant reduction in disease incidence and average CDS. The lower CDS in the *Esr1*$^{-/-}$ mice was also reflected as significant reductions in the severity and disease indices, SI and DI, respectively. Because the peak of disease in the wild-type and *Esr1*$^{+/-}$ mice occurred between days 18 and 25 after immunization, we stratified the clinical disease course into acute-early (day 7 to day 16) and chronic-late (day 17 to day 25) and reevaluated the clinical QTs (Table 2). No significant differences were seen for any of the parameters as a function of genotype during the acute-early phase of the disease. In contrast, *Esr1*$^{-/-}$ mice had significantly reduced CDS, SI, and DI compared to wild-type and *Esr1*$^{+/-}$ mice during the chronic-late phase of disease. These results indicate that Esr1 signaling plays a significant role in regulating the severity of the clinical signs seen in male mice during the chronic-late phase of the disease but not during the acute-early phase.

*EAE in B10.PL/SnJ, B10.PL-*Esr2$^{+/-}$*, and B10.PL-*Esr2^{tm1Unc} *Male Mice*

B10.PL/SnJ, B10.PL-*Esr2*$^{+/-}$ (*Esr2*$^{+/-}$), and B10.PL-*Esr2*^{tm1Unc} (*Esr2*$^{-/-}$) littermates were immunized with MBP_{Ac1-11} + CFA + PTX, and monitored for clinical EAE (Figure 3). No significant difference was seen for disease incidence among the three genotypes (Table 3); however, significant differences were detected for average day of onset, peak score, CDS, SI, DI, and mortality. With the exception of the day of onset, the clinical QT values for *Esr2*$^{+/-}$ mice were significantly increased over wild-type and *Esr2*$^{-/-}$ mice. These results are consistent with heterosis at *Esr2* in the regulation of EAE in male mice. Stratification of the disease course into acute-early and chronic-late resulted in similar differences with respect to each of the clinical QTs studied (data not shown). Similar results were seen in male *Esr1*$^{+/-}$*Esr2*$^{+/-}$ double heterozygotes (Table 4). *Esr1*$^{+/-}$*Esr2*$^{+/-}$ mice developed significantly more severe disease with an earlier onset and greater mortality compared to wild-type controls. Taken together these data suggest that heterosis at *Esr2*$^{+/-}$ influences the severity of the clinical signs throughout the course of the disease.

Table 3. Susceptibility to MBP_{Ac1-11}-Induced EAE in B10.PL/SnJ, B10.PL-*Esr2*$^{+/-}$, and B10.PL-*Esr2*^{tm1Unc} Male Mice

Genotype	Incidence	Onset	Peak score	SI	CDS	DI	Mortality
B10.PL/SnJ	22/24	14.5 ± 4.9*	3.9 ± 1.7	2.8 ± 1.4	30.7 ± 20.9	1.5 ± 1.0	1/24
Esr2$^{+/-}$	29/32	10.8 ± 2.9*	5.4 ± 0.6†	4.3 ± 1.0†	57.6 ± 27.4†	3.1 ± 1.5†	8/32
Esr2$^{-/-}$	17/18	12.4 ± 4.2*	4.5 ± 1.3	3.1 ± 1.4	37.7 ± 22.6	2.0 ± 1.2	1/18
	χ^2 = 0.23	F = 5.38	F = 9.41	F = 10.00	F = 9.26	F = 11.03	χ^2 = 6.38
P value	0.89	0.007	<0.001	<0.001	<0.001	<0.001	0.04

*Significance of differences based on one-way multiple comparisons with Tukey's and Hsu's family error rates at 0.1: wild-type > *Esr2*$^{+/-}$; wild-type = *Esr2*$^{-/-}$; and *Esr2*$^{+/-}$ = *Esr2*$^{-/-}$.

†Significance of differences based on one-way multiple comparisons with Tukey's and Hsu's family error rates at 0.1: *Esr2*$^{+/-}$ > *Esr2*$^{-/-}$ = wild-type.

Table 4. Susceptibility to MBP_{Ac1-11}-Induced EAE in B10.PL/SnJ and B10.PL-*Esr1*$^{+/-}$*Esr2*$^{+/-}$ F_1 Hybrid Male Mice

Genotype	Incidence	Onset	Peak score	SI	CDS	DI	Mortality
B10.PL/SnJ	22/22	15.8 ± 5.6	3.9 ± 1.9	2.7 ± 1.1	44.5 ± 21.3	2.9 ± 1.1	3/22
Esr1$^{+/-}$*Esr2*$^{+/-}$	25/25	12.0 ± 3.4	5.4 ± 0.8	4.1 ± 1.3	74.4 ± 32.5	3.9 ± 1.7	11/25
P value		0.006*	0.001	0.008	0.008	0.001	0.001†

*Significance of the differences between the means was determined using the Student's *t*-test with unequal sample sizes.
†Significance of the difference in mortality was determined by chi-square test with one degree of freedom: $\chi = 10.62$.

Histological EAE in B10.PL/SnJ, Heterozygous, B10.PL-Esr1^{tm1Unc}*, and B10.PL*-Esr2^{tm1Unc} *Male Mice*

Histopathological assessment of each of the previously defined QTs[28] was performed at day 25 after immunization (Figure 4). Briefly, these represent the overall severity of the lesions observed, extent and degree of myelin loss and tissue injury (swollen axon sheaths, swollen axons, and reactive gliosis), severity of the acute inflammatory response (predominantly neutrophils), and severity of the chronic inflammatory response (lymphocytes/macrophages). No significant differences were seen for any of the histopathological QTs studied in the brain or SC as a function of either *Esr1* (Figure 5) or *Esr2* genotype (Figure 6). Similar results were observed for the histopathological QTs in the brains and SCs between wild-type and *Esr1*$^{+/-}$ *Esr2*$^{+/-}$ double heterozygotes (data not shown).

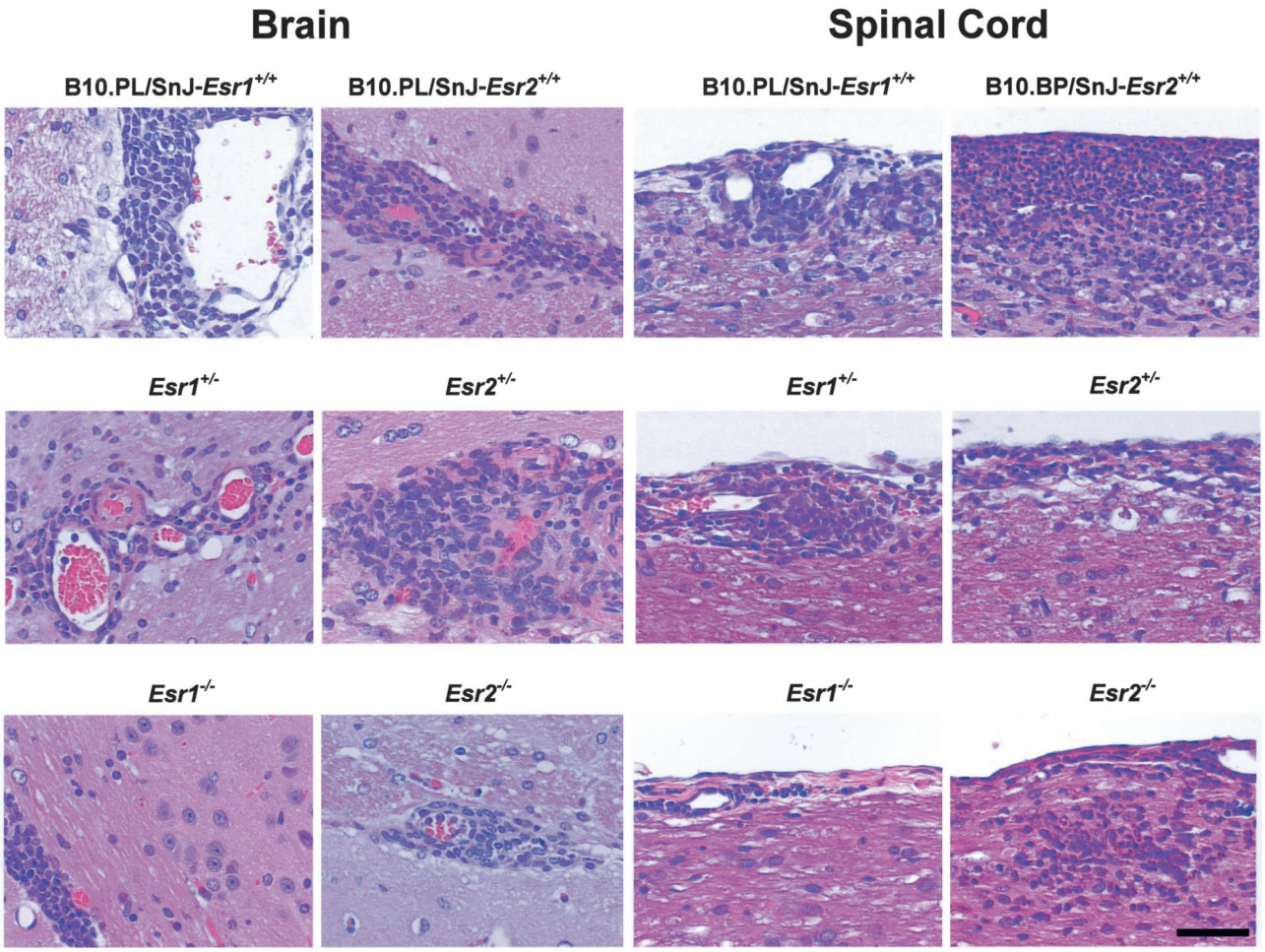

Figure 4. Histopathological lesions in the brains and SCs of male B10.PL/SnJ wild-type *Esr1* and *Esr2* line mice, *Esr1* and *Ers2* heterozygotes, and *Esr1* and *Esr2* knockout mice 25 to 26 days after injection. Brain lesions consisted of mononuclear cell (lymphocyte and macrophages) perivascular inflammation at the level of the external capsule (white matter myelinated tracts) of the cerebrum. Lesions in the SC consisted of a mixed inflammatory cell response consisting of neutrophils and mononuclear cells (lymphocyte and macrophages) in the pia mater and subjacent neuroparenchymal white matter (myelinated tracts). H&E stain. Scale bar, 50 μm (applies to all images).

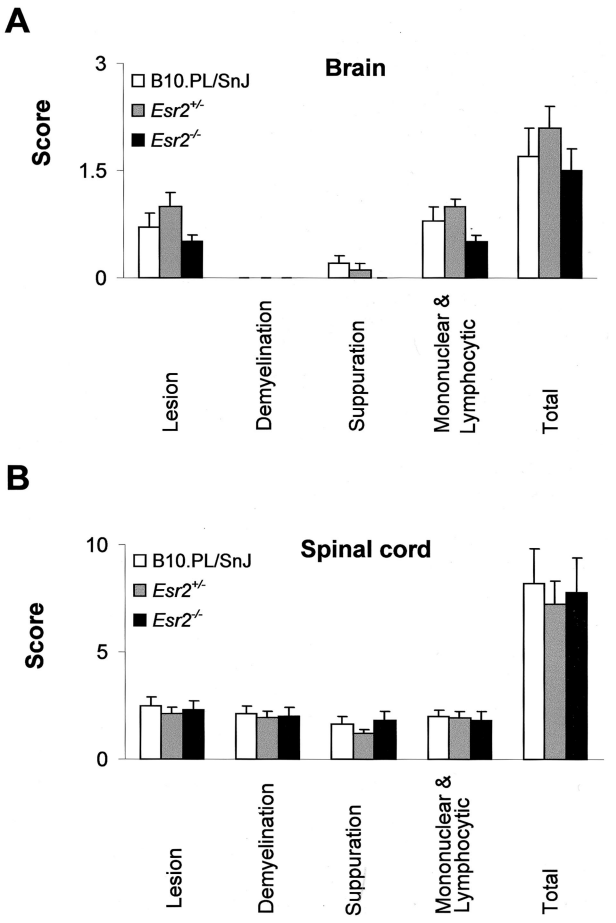

Figure 5. Histopathological QTs in the brains (**A**) and SCs (**B**) of B10.PL/SnJ, B10.PL-*Esr1*$^{+/-}$, and B10.PL-*Esr1*$^{-/-}$ male mice. Sections were stained with H&E for routine evaluation and Luxol fast blue-periodic acid-Schiff reagent for demyelination. Sections from representative areas were scored in a semiquantitative manner on a scale from 0 to 5 for each of the previously defined histopathological QTs.[28] Data are expressed as the mean ± SD. The total score is the average of the sum of the individual trait values.

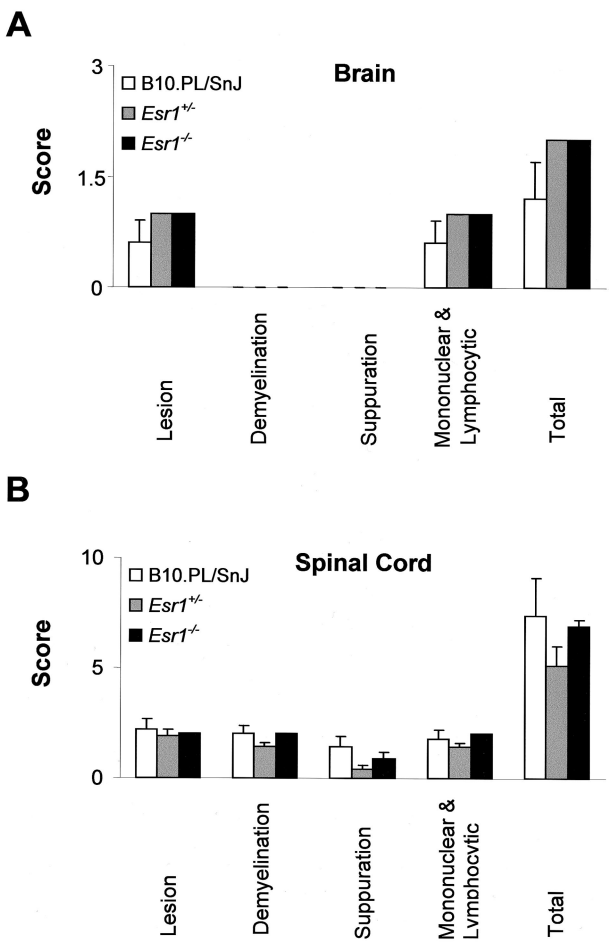

Figure 6. Histopathological QTs in the brains (**A**) and SCs (**B**) of B10.PL/SnJ, B10.PL-*Esr2*$^{+/-}$, and B10.PL-*Esr2*$^{-/-}$ male mice. Sections were stained with H&E for routine evaluation and Luxol fast blue-periodic acid-Schiff reagent for demyelination. Sections from representative areas were scored in a semiquantitative manner on a scale from 0 to 5 for each of the previously defined histopathological QTs.[28] Data are expressed as the mean ± SD. The total score is the average of the sum of the individual trait values.

*T-Cell Proliferative Responses and Cytokine/Chemokine Production by B10.PL/SnJ, Heterozygous, B10.PL-*Esr1^{tm1Unc}*, and B10.PL-*Esr2^{tm1Unc} *Mice*

A variety of T-cell parameters were examined in an effort to delineate a mechanistic basis for the inhibition of chronic-late phase EAE in *Esr1*$^{-/-}$ mice and the heterotic effect at *Esr2*. Significant difference in the *in vitro* T-cell proliferative responses to MBP_{Ac1-11} as a function of either the *Esr1* (Figure 7A) or *Esr2* (Figure 7B) genotype were not seen. In contrast, cytokine/chemokine production differed significantly. Of the cytokines studied, no significant differences in secretion were seen for IL-2, IL-4, IL-5, IL-10, and IL-12 (data not shown). IFN-γ secretion was found to be significantly negatively regulated by *Esr1* (Figure 8A) but uninfluenced by *Esr2* (data not shown); whereas TNF-α (Figure 9A) and IL-6 (Figure 9B) secretion both exhibited classical *Esr2* gene dose responses. Interestingly, MCP-1 secretion displayed distinctively unique patterns of genotype-dependent regulation by *Esr1* and *Esr2*. MCP-1 production in *Esr1*$^{+/-}$ mice was significantly less than either wild-type or *Esr1*$^{-/-}$ mice (Figure 8B); whereas wild-type mice produced significantly less MCP-1 than Esr2$^{+/-}$ and *Esr2*$^{-/-}$ mice. As with clinical disease, these results are consistent with the existence of ER-mediated regulation of TNF-α, IL-6, and MCP-1 secretion by encephalitogen-specific $CD4^+$ T cells in male mice.

*EAE in Reciprocal Radiation Bone Marrow Chimeras between B10.PL/SnJ and B10.PL-*Esr2$^{+/-}$ *Male Mice*

Reciprocal and control hematopoietic radiation bone marrow chimeras between wild-type and *Esr2*$^{+/-}$ male mice were constructed to map the functional component mediating the heterotic effect at *Esr2* to either the hematopoietic or nonhematopoietic cellular compartments (Table 5). Wild-type → wild-type and *Esr2*$^{+/-}$ → wild-type mice exhibited delayed onset and less severe disease as determined by SI, CDS, and DI compared to wild-type → *Esr2*$^{+/-}$ and *Esr2*$^{+/-}$ → *Esr2*$^{+/-}$ mice. These results in-

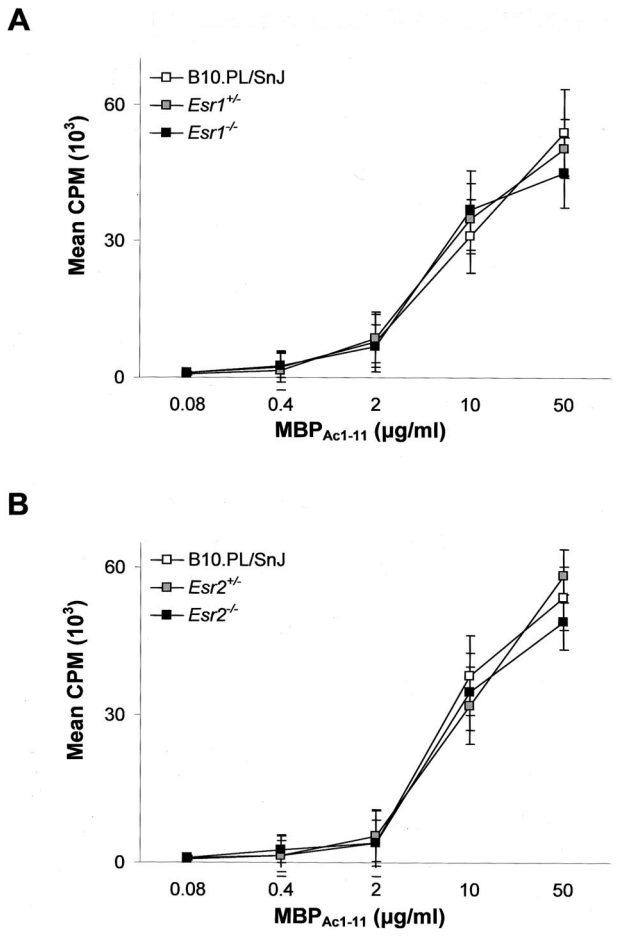

Figure 7. MBP_{Ac1-11}-specific T-cell proliferative responses for *Esr1* line mice (**A**) and *Esr2* line mice (**B**). Data are expressed as mean cpm ± SD based on triplicate wells for three mice.

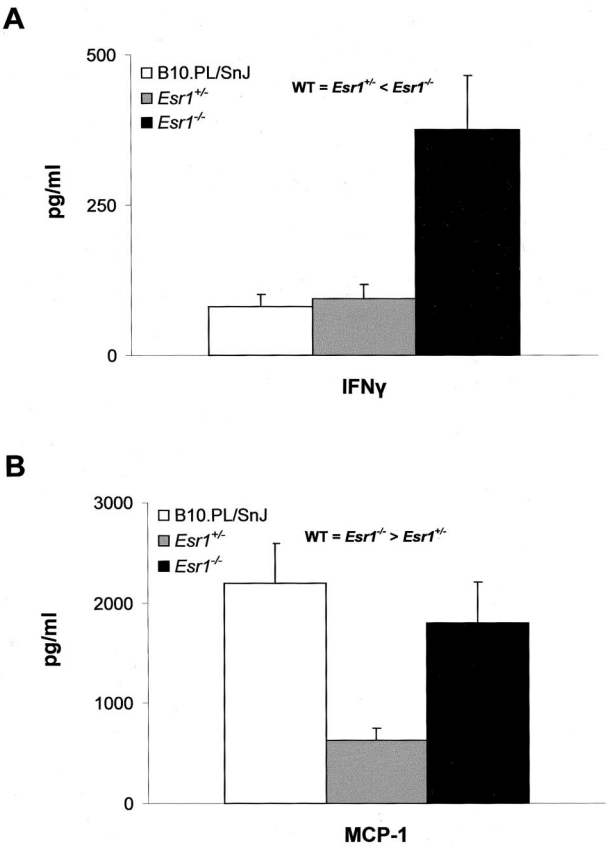

Figure 8. IFN-γ (**A**) and MCP-1 (**B**) secretion by MBP_{Ac1-11}-specific T cells from B10.PL/SnJ (□), B10.PL-*Esr1*$^{+/-}$ (▨), and B10.PL-*Esr1*$^{-/-}$ (■). Cytokine production was determined by either enzyme-linked immunosorbent assay or cytometric bead assay.

dicate that the heterotic effect at *Esr2* maps to the non-hematopoietic compartment.

Discussion

The function of estrogens and iERs has been well documented in female reproduction[16,17] and these ligand receptor pairs are believed to play a role in the sexual dimorphism observed in autoimmune diseases such as multiple sclerosis[34] and its animal model EAE.[26–28] The fact that Esr1 is required for normal thymic development and maintenance of optimal T lymphopoiesis[21,22] and in regulating B-cell development[9,10] in male mice, combined with the observation that males are equally susceptible to estrogen treatment in preventing and modulating disease susceptibility,[35] suggest that iERs may play a significant role in regulating autoimmune disease of the CNS in males. In this study we demonstrate that both *Esr1* and *Esr2* play unique roles in modulating clinical EAE in male mice.

Male *Esr1*$^{-/-}$ mice exhibit less severe disease during the chronic-late phase of the disease course compared to wild-type and *Esr1*$^{+/-}$ mice, indicating either that Esr1 signaling potentiates and enhances late clinical signs, or that its absence leads to increased expression of immunosuppressive factors. The results of the analysis of the cytokine/chemokine secreted by encephalitogen-specific T cells from *Esr1*$^{-/-}$ mice indicate that IFN-γ secretion is negatively regulated by Esr1 signaling. This stands in contrast to the report that estrogen exerts a direct stimulatory effect on IFN-γ gene expression *in vitro*.[36] However, Cenci and colleagues,[37] recently demonstrated that ovariectomy specifically up-regulates IFN-γ production by Th1 cells *in vivo*. The negative regulation of IFN-γ secretion by Esr1 provides a mechanistic basis for the inhibition of chronic-late phase disease in *Esr1*$^{-/-}$ male mice in that a considerable body of evidence exists indicating that IFN-γ has potent disease-inhibitory activity in EAE.[38–41] It is worth noting that the negative inhibition of IFN-γ production by encephalitogen-specific T cells may be indirect because of the effects of Esr1 signaling in other hematopoietic cells, particularly dendritic cells,[42,43] and may even require direct cell-cell contact.[44]

In comparison to *Esr1*$^{-/-}$ mice, *Esr2*$^{-/-}$ mice are phenotypically identical to wild-type mice with respect to clinical and histological disease. However, a significant heterotic effect at *Esr2* is seen; *Esr2*$^{+/-}$ mice develop more severe disease compared to wild-type and *Esr2*$^{-/-}$ mice. Heterosis for susceptibility to EAE is not without precedence and is seen in several F_1 hybrid combinations.[45] Importantly, heterosis for EAE is not seen in

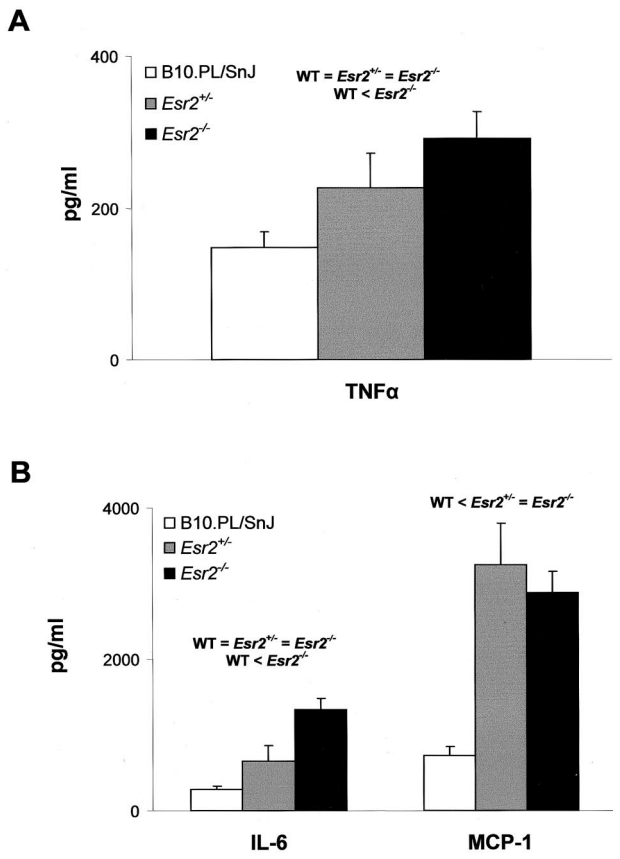

Figure 9. TNF-α (**A**) and IL-6 and MCP-1 (**B**) secretion by MBP_{Ac1-11}-specific T cells from B10.PL/SnJ (□), B10.PL-*Esr2*$^{+/-}$ (▨), and B10.PL-*Esr2*$^{-/-}$ (■). Cytokine production was determined by either enzyme-linked immunosorbent assay or cytometric bead assay.

(C57BL/6 × 129) F_1 hybrid mice[41,46] indicating that the heterotic effect at *Esr2*$^{+/-}$ is a function of the *Esr2* genotype rather than 129-strain alleles at linked loci within the congenic interval. The heterotic effect does not seem to be because of increased T-cell proliferative responses or proinflammatory cytokine/chemokine secretion because neither parameter exhibits a significant increase in *Esr2*$^{+/-}$ mice over wild-type or *Esr2*$^{-/-}$ mice. This is consistent with the results of the radiation bone marrow chimera studies indicating that the heterotic effects maps to the nonhematopoietic cellular compartment. Additionally, our results suggest that single hit germ line or somatic mutations affecting Esr2 activity have the potential to significantly impact autoimmune disease of the CNS. In this regard, a growing body of evidence indicates that haploinsufficiency at a number of tumor suppressor genes in which a single allele is mutated or disrupted is sufficient to lead to tumorigenesis without inactivation of the second allele.[47]

The mechanism whereby heterosis at *Esr2* influences the severity of the clinical signs associated with EAE as a function of the parenchyma, which includes cells in the CNS, is unknown but may be occurring prenatally, postnatally, or both.[16,17] Esr2 has been shown to be required for normal brain development[48,49] and recent immunohistochemical staining and expression analysis during postnatal development revealed significant cell-specific changes in the expression of *Esr2* in cells of the lumbar tract of the ventral SC,[50] the location of the earliest and most significant events that correlate with the severity of the clinical signs of EAE.[51–55] *Esr1* expression was greater during early development, declining with age whereas *Esr2* expression was minimal early, increasing with age and peaking at postnatal day 25. Co-localization studies revealed that at postnatal day 25, *Esr1* and *Esr2* are simultaneously expressed in astrocytes and oligodendrocytes within this region of the SC. Given that the binding of agonist facilitates an activating conformational change that permits iERs to form either α/α, α/β, or β/β dimers mediating receptor functions,[56,57] the possibility exists that the heterotic effect at *Esr2* is the result of a genotype-based imbalance in the relative proportion of α/α, α/β, or β/β dimer formation.

Heterosis in CNS development is not without precedence. Heterosis for brain myelin content has been reported for a variety of constituents including cerebroside, G_{M1} ganglioside, and myelin proteins including 2′,3′-cyclic nucleotide, 3′-phosphohydrolase (CNPase), and MBP,[58–63] the target encephalitogen used in these studies. Additionally, the expression of myelin proteins, including MBP, CNPase, myelin-associated glycoprotein, and proteolipid protein, all of which are either encephalitogenic or autoantigenic,[64] are regulated by steroid hormones.[65–70] Similarly, steroid hormones determine the number of neurons in the sexually dimorphic motor nucleus of the lumbar SC.[71–73] Thus, it is tempting to hypothesize that heterosis at *Esr2* for increased severity of clinical signs of EAE in male mice may be related to the regulation of the expression of SC myelin constituents or the number of SC motor neurons during development. In this regard, B10.S SC homogenate is significantly less effective at eliciting EAE in SJL/J mice compared to autologous SJL/J SC homogenate (unpublished data).[74]

Table 5. Susceptibility to MBP_{Ac1-11}-Induced EAE in Radiation Bone Marrow Chimeras between B10.PL/SnJ and B10.PL-*Esr2*$^{+/-}$ Male Mice

Genotype	Incidence	Onset	Peak score	SI	CDS	DI	Mortality
B10.PL/SnJ → B10.PL/SnJ	6/6	14.2 ± 3.3	4.5 ± 1.3	2.8 ± 1.1	36.4 ± 15.0	1.9 ± 0.8	0/6
B10.PL/SnJ → *Esr2*$^{+/-}$	6/6	9.7 ± 1.5*	5.6 ± 0.4	4.6 ± 0.9*	74.5 ± 15.0*	3.9 ± 0.8*	2/6
Esr2$^{+/-}$ → B10.PL/SnJ	6/6	13.7 ± 3.2	4.1 ± 1.7	3.6 ± 1.4	38.4 ± 22.8	2.0 ± 1.2	1/6
Esr2$^{+/-}$ → *Esr2*$^{+/-}$	6/6	9.8 ± 1.6*	5.3 ± 0.5	4.5 ± 1.0*	68.6 ± 19.7*	3.6 ± 1.0*	1/6
		$F = 5.54$	$F = 2.34$	$F = 5.26$	$F = 6.98$	$F = 7.26$	$\chi^2 = 2.40$
P value		0.006	0.10	0.008	0.002	0.002	0.49

*Significance of differences based on one way multiple comparisons with Tukey's and Hsu's family error rates of 0.1: wild-type → wild-type = *Esr2*$^{+/-}$ → wild-type ≠ wild-type → *Esr2*$^{+/-}$ and *Esr2*$^{+/-}$ → *Esr2*$^{+/-}$.

This is consistent with the concept that susceptibility to EAE, and by inference multiple sclerosis, may in part be determined by genes controlling myelin development, organization, and function.[75,76]

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