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# Estrogen Enhances Susceptibility to Experimental Autoimmune Myasthenia Gravis by Promoting Type 1-Polarized Immune Responses[1]

**Laurent Delpy, Victorine Douin-Echinard, Lucile Garidou, Corinne Bruand, Abdelhadi Saoudi, and Jean-Charles Guéry[2]**

**Myasthenia gravis (MG) is an organ-specific autoimmune disease caused in most cases by autoantibodies against the nicotinic acetylcholine receptor (AChR). It is now well documented that many autoimmune diseases, including MG, are more prevalent in women than in men, and that fluctuations in disease severity occur during pregnancy. These observations raise the question of the potential role of sex hormones, such as estrogens, as mediators of sex differences in autoimmunity. In the present study, we have analyzed the effect of 17$\beta$-estradiol (E2) on the pathogenesis of experimental autoimmune myasthenia gravis (EAMG), an animal model of MG. We show that treatment with E2 before Ag priming is necessary and sufficient to promote AChR-specific Th1 cell expansion in vivo. This time-limited exposure to E2 enhances the production of anti-AChR IgG2a$^b$ (specific for b allotype; e.g., B6) and IgG2b, but not IgG1, and significantly increases the severity of EAMG in mice. Interestingly, the E2-mediated augmentation in AChR-specific Th1 response correlates with an enhanced production of IL-12 by splenic APCs through the recruitment of CD8$\alpha^+$ dendritic cells. These data provide the first evidence that estrogen enhances EAMG, and sheds some light on the role of sex hormones in immune responses and susceptibility to autoimmune disease in women.** *The Journal of Immunology,* 2005, 175: 5050–5057.

Myasthenia gravis (MG)[3] is a T cell-dependent B cell-mediated organ-specific autoimmune disease caused in most cases by autoantibodies against the nicotinic acetylcholine receptor (AChR) (1). Several mechanisms may account for the pathogenicity of anti-AChR autoantibodies at the neuromuscular junction of skeletal muscle. They include complement-mediated destruction of the postsynaptic membrane and accelerated internalization and degradation of functional AChR resulting in chronic muscular weakness (2). Experimental autoimmune myasthenia gravis (EAMG) can be induced in susceptible mouse and rat strains by immunization with AChR from the electric organs of *Torpedo californica* (tAChR). Animals develop a T cell-dependent Ab response against tAChR that cross-reacts with their own receptor, resulting in a neuromuscular disease with clinical symptoms resembling human MG. The role of Th1/Th2 balance in the development of EAMG and MG is still a

matter of debate. In rats and humans, several studies described the involvement of both Th1 and Th2 responses (3–5). In mice, the importance of Th1 cytokines rather than the prototypic Th2 cytokine, IL-4, in the pathogenesis of EAMG is well demonstrated (6–8). However, it has also been shown that other type 2 cytokines such as IL-5 and IL-10 could influence the development of this disease (9–11).

It is now well documented that many autoimmune diseases, including MG, are more prevalent in women than in men (12). Within MG patients, the early onset form of the disease occurs with a female to male ratio of 3:1 (2). Furthermore, fluctuations in disease severity have been reported during pregnancy in MG patients (13, 14). Generally, in pregnant women, the first trimester of gestation and the first postpartum month seem to be the most critical periods for MG exacerbations (13, 14). Disease exacerbations have also been reported during pregnancy in systemic lupus erythematosus (SLE) (15, 16), an immune complex-mediated autoimmune disease. In contrast, beneficial effect of pregnancy on clinical symptoms has been observed for cell-mediated inflammatory autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis (MS) (17–19). Such observations have suggested that sex steroid hormones such as estrogens or progesterone could modulate disease activity through their action on the immune system (20). In agreement with the clinical data in human, studies of estrogen's effects on murine models of SLE have shown that 17$\beta$-estradiol (E2) administration accelerates disease onset and severity in NZB/NZW F$_1$ (21, 22) and MRL/*lpr* mouse strains (23). By contrast, administration of estrogens in collagen-induced arthritis (24, 25) or experimental autoimmune encephalomyelitis (EAE) has been shown to inhibit disease development (26, 27). To date, the underlying mechanisms responsible for these paradoxical effects of estrogens on autoimmune diseases are still ill defined.

To investigate mechanisms by which sex hormones could contribute to the development of MG, we have analyzed the effect of

[3] Abbreviations used in this paper: MG, myasthenia gravis; AChR, acetylcholine receptor; DC, dendritic cell; E2, 17$\beta$-estradiol; EAMG, experimental autoimmune myasthenia gravis; ER, estrogen receptor; LNC, lymph node cell; MS, multiple sclerosis; RA, rheumatoid arthritis; SAC, *Staphylococcus aureus* cell; SLE, systemic lupus erythematosus; tAChR, AChR from electric organs of *Torpedo californica*.

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estrogen administration on AChR-specific T and B cell responses and on the outcome of EAMG in mice. We show that hormonal treatment limited to the 3 wk before immunization enhances AChR-specific Th1 cell expansion and anti-AChR IgG2a[b] (specific for b allotype; e.g., B6) and IgG2b production, and significantly increases the severity of EAMG in C57BL/6 (B6) mice. Interestingly, we demonstrate that the E2-mediated increase in Ag-specific Th1 cell development correlates with an enhanced capacity of splenic APCs to produce IL-12 through the recruitment of CD8α^{+} dendritic cells (DCs). These data provide the first evidence for a role of estrogens in the pathogenesis of EAMG.

## Materials and Methods

### Mice and hormonal treatment

C57BL/6 (B6) mice were purchased from the Centre d'Elevage R. Janvier and maintained in our animal facilities under specific pathogen-free conditions. One- to 3-mo-old female mice were used in all experiments. For estrogen hormone administration, 3-mm pellets (Innovative Research of America) containing 0.01, 0.1, 0.25, or 2.5 mg of E2 were implanted s.c. on the animal back at 4–5 wk of age. These pellets provide continuous controlled release of a constant level of hormone over a period of 60 days. When indicated, these pellets were removed or implanted by surgical intervention the day of immunization. Serum estrogen levels were measured using a commercially available RIA kit (Beckman-Coulter). All of the protocols used have been approved by our institutional review board for animal experimentation.

### Purification of tAChR and induction of EAMG

tAChR was purified from electric organs of *Torpedo californica* by affinity chromatography on a conjugate of neurotoxin coupled to agarose, as previously described (28). To induce EAMG, 8-wk-old mice were immunized with 10 $\mu$g of tAChR emulsified in CFA (Sigma-Aldrich) in a total volume of 100 $\mu$l, injected s.c. in the tail base. Four weeks after the first immunization, mice were boosted with 10 $\mu$g of tAChR emulsified in IFA (Sigma-Aldrich) in a total volume of 200 $\mu$l, injected in the flanks and at the tail base. Control mice received an equal volume of PBS in CFA (100 $\mu$l) or IFA (200 $\mu$l).

### Measurement of muscle AChR content

Three weeks after the second immunization, the concentration of AChR present in total body musculature was measured in muscle detergent extract by RIA, as previously described (3). Briefly, the frozen carcasses were homogenized, and membrane-bound proteins were extracted with PBS containing 2% Triton X-100 (Sigma-Aldrich). Aliquots (250 $\mu$l) of each extract were labeled in triplicate with $2 \times 10^{-9}$ M $^{125}$I-labeled α-bungarotoxin (Amersham; sp. act., >150 Ci/mmol) incubated overnight with an excess of rat anti-AChR, and precipitated by goat or sheep anti-rat IgG. The concentration of AChR in muscle was expressed as moles of $^{125}$I-labeled α-bungarotoxin precipitated per gram of muscle, and the percentage of mouse AChR contents was calculated by comparison with that in control adjuvant-immunized mice.

### RIA for serum anti-mouse AChR Abs

Individual mouse sera were prepared from bleeding collected the day of secondary immunization and 3 wk later when mice were killed to measure muscle AChR loss. The concentration of Abs reactive with mouse AChR was determined in individual sera by RIA, as previously described (29). Briefly, mouse AChR was extracted from leg muscles and labeled with $2 \times 10^{-9}$ M $^{125}$I-labeled α-bungarotoxin (Amersham). A dilution range of serum samples was incubated overnight with 200 $\mu$l of labeled mouse AChR. Ab-AChR complexes were captured by adding an excess of rabbit anti-mouse IgG (Sigma-Aldrich) or protein G (Amersham). The radioactivity of the complexes was measured in a gamma counter. Values of $^{125}$I-labeled α-bungarotoxin-AChR pelleted in the presence of normal mouse serum were subtracted from the assay values. Corrections for interassay variability were made based on serial dilutions of an EAMG standard control serum pool tested in each assay. The Ab titers were expressed as moles of $^{125}$I-labeled α-bungarotoxin binding sites precipitated per liter of serum.

### ELISA for anti-tAChR Ab isotypes

Microtiter plates (Falcon 3012; BD Discovery Labware) were coated overnight at 4°C with 1 $\mu$g/ml tAChR in PBS and incubated with serial dilutions of individual sera. Bound total IgG and IgG2b were revealed using

biotin goat anti-mouse isotype-specific Abs (Southern Biotechnology Associates); bound IgG1 and IgG2a[b] were detected using HRP-conjugated rat anti-mouse mAb (LO/IMEX) and biotin-conjugated mouse anti-mouse mAb 5.7 (BD Pharmingen), respectively. The bound biotinylated mAbs were revealed by addition of preformed streptavidin-biotin-peroxidase complexes (Amersham) for 30 min at 37°C, and bound peroxidase was detected, as described (30). Each serum, from bleeding collected the day of priming (day 0) and 14 days after (day 14), was tested in duplicate and was assessed at four different dilutions. Standard curves were generated using pooled anti-tAChR sera, and results are expressed as arbitrary U/ml.

### Cell culture

Erythrocyte-depleted spleen cells and popliteal and inguinal lymph node cells (LNC) were cultured at indicated concentrations ($3–6 \times 10^5$ cells/well) in 96-well culture plates (Costar) in synthetic HL-1 medium (Hycor) supplemented with 2 mM L-glutamine (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Eurobio). Cultures were incubated (for 1–3 days) in the presence of various concentrations of tAChR protein, tAChR α146–162 peptide (Neosystem), or a set of 16-mer synthetic peptides overlapping by 12 residues and encompassing the extracellular domain (aa 1–210) of tAChR α-subunit (kindly provided by L. Adorini, Bioxell, Milano, Italy). For spleen cell stimulation, cells were cultured with *Staphylococcus aureus* cells (SAC) (Pansorbin cells; Calbiochem) at a final dilution of 1/5000 plus 5 ng/ml mouse rIFN-γ (Sigma-Aldrich).

### Cytokine and proliferation

The cytokine analysis, supernatants were collected after 24- to 72 h of culture. IFN-γ, TNF-α, IL-4, IL-5, and IL-10 were quantified by sandwich ELISA, as described (31, 32). IL-12p40 and p70 subunits were measured using for coating mAb C15.6 (BD Pharmingen) and mAb C18.2 (eBiosciences), respectively, and for detection biotin-labeled anti-IL-12 mAb C17.8 (BD Pharmingen). IL-6 was measured using mAb MP5-20F3 for coating and biotin-labeled mAb MP5-32C11 for detection, both obtained from eBiosciences. Recombinant cytokines were used as standard (BD Pharmingen). For T cell proliferation assays, cells ($4.5 \times 10^5$ cells/well) were pulsed with 1 $\mu$Ci of [$^3$H]TdR (40 Ci/nmol; Radiochemical Centre) during the last 12 h of culture before harvesting on glass fiber filter. Incorporation of [$^3$H]TdR was measured by direct counting using an automated beta plate counter (MatrixTM 9600; Packard Instrument).

### Flow cytometric analysis

Cells were stained using FITC-, PE-, biotin-, or allophycocyanin-conjugated anti-CD4, CD19, CD8α, CD11c, CD11b, CD44, and CD62L, followed by streptavidin-CyChrome, all purchased from BD Pharmingen. For DC population analysis, spleen cells were homogenized and digested for 30 min with 400 U/ml collagenase IV (Sigma-Aldrich) and 1 $\mu$g/ml DNase I (Roche) under continuous agitation at 37°C. For the intracellular analysis of cytokine synthesis, LNC were resuspended at $2 \times 10^6$ cells/ml in 10% FCS RPMI 1640 (Eurobio) medium and stimulated for 8 h with the immunodominant peptide α146–162 (3 $\mu$M) plus soluble anti-CD28 (37.51) mAb (5 $\mu$g/ml) (eBiosciences), in the presence of brefeldin A (Sigma-Aldrich) at a concentration of 10 $\mu$g/ml during the last 4 h. Cells were then harvested, washed, and stained using allophycocyanin-conjugated anti-CD4 mAb. Labeled cells were then fixed with 2% paraformaldehyde (Fluka). Intracytoplasmic staining using PE-conjugated anti-TNF-α, anti-IFN-γ, and anti-IL-4 (BD Pharmingen) was performed, as described (32). Extracellular staining was analyzed on viable cells, as determined by propidium iodide exclusion. LNC and DC analysis were performed on $3 \times 10^4$ CD4^{+} and CD11c^{+} cells, respectively. Data were collected on a FACS-Calibur (BD Biosciences), and analyzed using CellQuest software (BD Biosciences).

### Statistical analysis

Results are expressed as mean ± SEM, and overall differences between variables were evaluated by a two-tailed unpaired Student's *t* test using Prism GraphPad software.

## Results

### Dose-dependent effect of E2 administration on primary tAChR-specific T cell responses
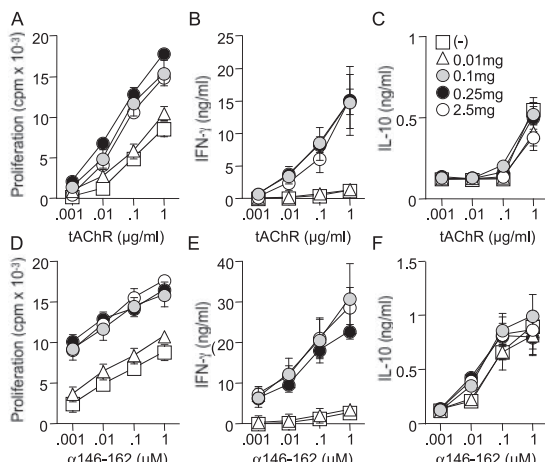
To evaluate the potential role of estrogens on EAMG, we first analyzed the effect of continuous E2 administration on tAChR-specific CD4 T cell responses. In B6 mice, tAChR-specific CD4 T
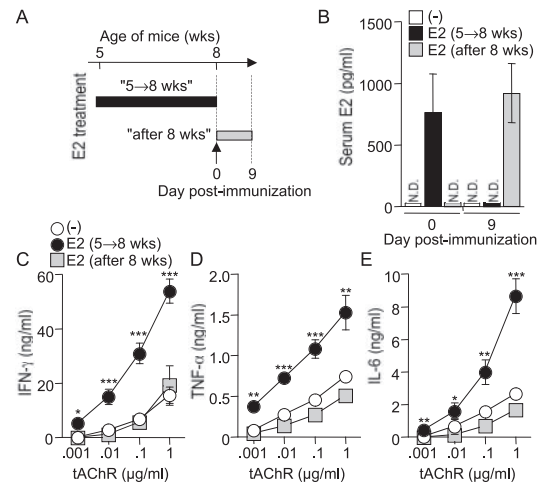
cells are mostly directed against the immunodominant epitope of the protein contained in the sequence 146–162 of the tAChR α-subunit presented by I-A[b] MHC class II molecules. As shown in Fig. 1, *A* and *D*, the proliferative response of CD4 T cells specific for tAChR or its immunodominant epitope α146–162 was strongly enhanced in mice treated with 0.1–2.5 mg of E2 pellets, whereas administration of 0.01 mg of pellets had no effect on T cell responsiveness. This increased lymphoproliferative response in E2-treated mice was associated with a dramatic up-regulation of IFN-γ production (Fig. 1, *B* and *E*). By contrast, IL-4 and IL-5 were not detected in all groups, and IL-10 production was similar in placebo- and E2-treated mice (Fig. 1, *C* and *F*, and data not shown). Because optimal effect of E2 was observed with pellets ranging from 0.1 to 2.5 mg, the intermediate dose of E2 (0.25 mg) was subsequently used throughout the study. To test whether E2 induces qualitative changes in the specificity of tAChR-reactive CD4 T cells, the T cell response to a set of 16-mer peptides overlapping by 12 aa encompassing the extracellular domain of tAChR α-subunit (region 1–210) was tested. We found that tAChR-specific CD4 T cell repertoire was mainly directed against the immunodominant determinant contained in the sequence α146–162 (data not shown).

*Limited exposure to E2 before priming is necessary and sufficient to promote optimal tAChR-specific Th1 cell expansion in vivo*

To determine the optimal condition of E2 administration for the induction of an enhanced Ag-specific Th1 cell response in vivo, we first evaluated whether the presence of the hormone at the time of T cell priming was required (Fig. 2*A*). To test this, E2 pellets were implanted in mice 3 wk before priming and surgically removed at the time of immunization, resulting in a rapid drop of hormone level to basal values (Fig. 2*B*). In these mice, E2 levels were already dramatically decreased (from 766.6 to <15 pg/ml) by day 3
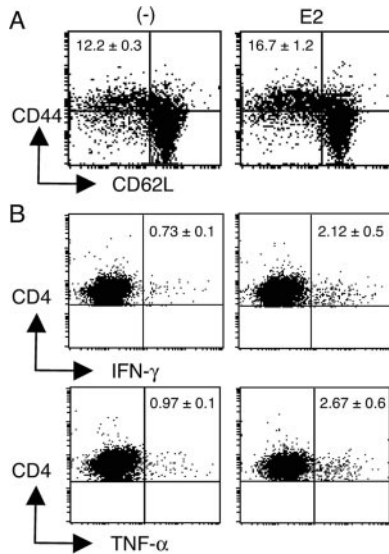


**FIGURE 2.** E2 administration before immunization is necessary and sufficient to enhance AChR-specific Th1 responses. *A*, B6 female mice were either left untreated (−) or implanted with 0.25 mg of E2 pellets at 5 wk of age. Three weeks later, E2 pellets were surgically removed from the E2 "5→8 wks" group. An additional group of mice was implanted with E2 pellets the day of immunization at 8 wk of age (E2 "after 8 wks"). All mice were immunized as described in Fig. 1. *B*, Serum was collected the day of immunization and the following 3 and 9 days and assessed for 17β-estradiol quantification by RIA (N.D., not detectable; <15 pg/ml). Draining LNC were harvested 9 days after immunization and stimulated as described in Fig. 1. IFN-γ (*C*), TNF-α (*D*), and IL-6 (*E*) production was assessed in 72-h culture supernatants by ELISA. Untreated mice are represented by □ and ○, 3-wk E2-treated mice (with pellet removal the day of immunization) by ■ and ●, and mice treated with E2 the day of immunization by ▨ (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$).

following removal of hormone pellets (data not shown). Results in Fig. 2, *C–E*, show that limited exposure to E2 during 3 wk before priming was sufficient to induce an enhanced production of the type 1 cytokines IFN-γ and TNF-α, as well as IL-6, by CD4 T cells specific for tAChR protein or α146–162 peptide (Fig. 2, *C–E*, and data not shown). In contrast, E2 administration at the time of immunization did not increase Ag-specific T cell responses (Fig. 2, *C–E*).

Next, we evaluated whether up-regulation in type 1 cytokine production was due to an enhanced expansion of Ag-specific CD4 T cells in draining lymph nodes in vivo. In Fig. 3*A*, we show that the frequency of memory/effector CD44^{high}CD62L^{low} CD4 T cells was significantly increased ($p = 0.011$) in LNC from E2-treated mice. To quantify Ag-specific effector Th1 cells ex vivo, lymph node CD4 T lymphocytes were stimulated with α146–162 peptide for 8 h and stained for intracellular cytokines. Data in Fig. 3*B* show that there was an overall 3-fold increase in the frequency of IFN-γ- or TNF-α-producing α146–162-specific CD4 T cells in E2-treated mice. In contrast, the Th2 cytokines IL-4 and IL-5 were undetectable in the culture supernatants or by intracellular staining (data not shown). Therefore, the E2-dependent enhanced production of type 1 cytokines is due to an increased expansion of Ag-specific memory/effector Th1 cells in vivo.

*Limited exposure to E2 before primary immunization to tAChR enhances EAMG and complement-fixing anti-AChR Abs*

To analyze the effect of estrogen administration on EAMG development, mice were treated with E2 during the 3 wk before the primary immunization with tAChR in CFA and challenged with the same Ag in IFA 1 mo later. Mice were killed 3 wk after the



**FIGURE 1.** Dose-dependent effect of E2 administration on AChR-specific CD4 T cell proliferation and IFN-γ production. Normal 4- to 5-wk-old B6 female mice were implanted with pellets containing various doses of E2 (0.01 mg/pellet, △; 0.1, 0.25, and 2.5 mg/pellet, light gray circles, ●, and ○, respectively) or left untreated (□). At 8 wk of age, mice were immunized s.c. in the hind footpads with 5 μg of tAChR in CFA. Draining LNC were harvested 9 days later and stimulated for 72 h with the indicated doses of tAChR (*A–C*) or a 16-mer peptide corresponding to the region 146–162 of tAChR α-subunit (α146–162) (*D–F*). Proliferation was assessed by pulsing LNC (4.5 × 10^{5} cells/well) with 1 μCi of [³H]TdR during the last 12 h of culture (*A* and *D*). IFN-γ (*B* and *E*) and IL-10 (*C* and *F*) secretion was tested by ELISA in 72-h LNC (6 × 10^{5} cells/well) culture supernatants.

**FIGURE 3.** E2 administration induces preferential expansion of memory/effector CD4 T lymphocytes. Normal 4- to 5-wk-old B6 mice were implanted with 0.25 mg of E2 pellets or left untreated. At 8 wk of age, E2 pellets were removed and mice were immunized as described in Fig. 1. Draining LNC were harvested 9 days later and stained for CD4, CD44, and CD62L (*A*). *B*, LNC were stimulated for 8 h with peptide $\alpha$146–162 (3 $\mu$M) plus soluble anti-CD28 (5 $\mu$g/ml) and stained for surface CD4 and intracellular cytokines (IFN-$\gamma$ and TNF-$\alpha$), as described in *Materials and Methods*. Analysis was performed on $3 \times 10^4$ CD4$^+$ cells and expressed as mean $\pm$ SEM of four mice per group. Data are from one representative experiment of two performed.

second immunization to perform quantification of endogenous AChR in total body musculature. AChR contents were significantly reduced in most of the tAChR-immunized mice as compared with mice primed with CFA alone (Fig. 4*A*). Interestingly, muscle AChR loss was significantly more pronounced in mice that received E2 before the primary tAChR immunization as compared
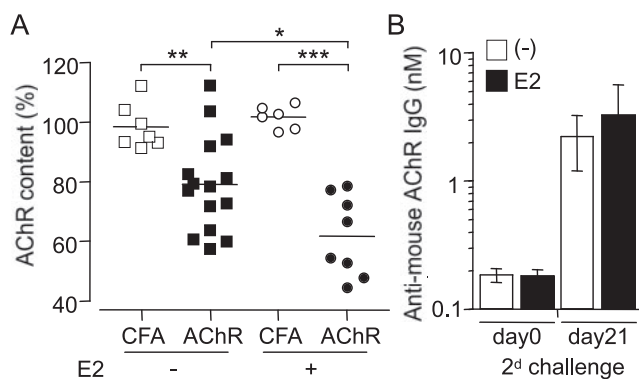


**FIGURE 4.** E2 administration before immunization enhances severity of EAMG. Normal 4- to 5-wk-old B6 female mice were implanted with 0.25 mg of E2 pellets or left untreated. In 8-wk-old mice, E2 pellets were removed and EAMG induction was performed by immunizations with 10 $\mu$g of tAChR, as described in *Materials and Methods*. Twenty-one days after the second immunization, mice were killed and endogenous AChR quantification was measured by RIA on whole body musculature. Data were expressed as the percentage of the AChR content of CFA-immunized age-matched control mice (*A*). The concentration of IgG Abs reactive with mouse AChR was determined by RIA in individual sera from days 0 and 21 after the second immunization (*B*). Data are pooled from two experiments; a $p$ value $<0.05$ was considered as significant (*, $p = 0.017$; **, $p < 0.01$; ***, $p < 0.001$).

with placebo-treated mice (percentage of AChR content, $61.7 \pm 4.8$ and $79.2 \pm 4.1$, respectively, $p = 0.017$). The enhanced AChR loss in E2-pretreated mice was not due to a direct effect of the hormone on the expression of endogenous AChR because similar AChR contents were measured in adjuvant-immunized mice, treated or not with E2 (Fig. 4*A*).

Next, we determined the effect of E2 treatment on the production of AChR-specific autoantibodies. Data in Fig. 4*B* show that serum anti-mouse AChR IgG concentrations were strongly enhanced after the second challenge in both groups of mice. Although E2-treated animals exhibited a higher concentration of autoantibodies than the untreated ones (respectively 3.23 and 2.20 nM), the difference in anti-mouse AChR IgG levels in both groups was not statistically significant. Moreover, as previously described (33, 34), the serum concentration of AChR-specific Abs did not correlate with AChR loss in total body musculature (data not shown). To evaluate whether E2 treatment induced qualitative changes in complement-fixing Ab titers, we analyzed the anti-tAChR IgG subclasses. Data in Fig. 5*A* show that E2-treated mice exhibited an overall 2-fold increase ($p < 0.01$) of anti-tAChR IgG as compared with untreated animals. Interestingly, this up-regulation was mainly due to enhanced production (3- to 4-fold increase) of complement-fixing IgG2a$^b$ (Fig. 5*B*) and IgG2b (Fig. 5*C*) isotypes in E2-treated mice. By contrast, similar amounts of anti-tAChR IgG1 were measured in untreated and E2-treated animals (Fig. 5*D*). These results strongly suggest that the enhanced AChR loss in E2-pretreated mice is due to an increase production of pathogenic complement-fixing Abs, which is in agreement with the preferential expansion of tAChR-specific polarized Th1 cells.

*Enhanced tAChR-specific Th1 expansion is associated with an increased IL-12 production by splenic APCs in E2-treated mice*

To further investigate the basis for the E2-mediated increase in EAMG susceptibility, we then analyzed the spleen cell populations 14 days after tail base immunization with tAChR. As shown in Table I, E2-preteated mice exhibited a significant increase in spleen cellularity as compared with placebo-treated mice (respectively $150 \times 10^6$ and $105 \times 10^6$ cells/spleen). This was mainly due to an E2-mediated 1.5-fold increase in the number of B cells. In contrast, absolute numbers of CD4$^+$ and CD8$^+$ T lymphocytes were not altered by E2 treatment (Table I). Consistent with our data in Fig. 2, AChR-specific Th1 response by splenic CD4 T cells was also strongly enhanced in E2-treated mice (data not shown).
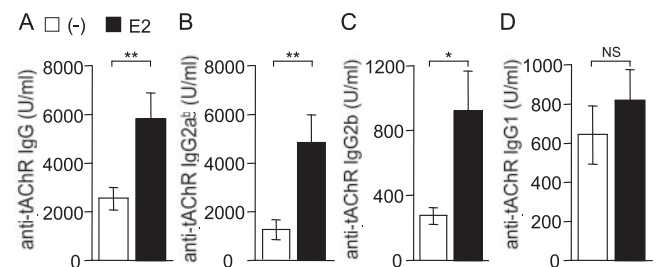


**FIGURE 5.** Limited exposure to E2 enhances the production of complement-fixing anti-tAChR Ab isotypes. Normal 4- to 5-wk-old B6 mice were implanted with 0.25 mg of E2 pellets or left untreated. At 8 wk of age, E2 pellets were removed and mice were immunized in the tail base with 5 $\mu$g of tAChR in CFA. Sera were collected 14 days after immunization, and the concentration of total IgG (*A*), IgG2a$^b$ (*B*), IgG2b (*C*), and IgG1 (*D*) reactive with tAChR was determined by ELISA in individual sera from untreated ($\square$) or E2-treated ($\blacksquare$) mice. Data are pooled from three experiments and expressed as arbitrary U/ml serum. A $p$ value $<0.05$ was considered as significant (*, $p = 0.013$ for $\gamma$2b isotypes; **, $p < 0.01$).

Table I. *Analysis of spleen cell populations in control and E2-treated AChR-immunized mice*

| | Control | E2 | *p* Value[a] | Fold Increase |
|---|---|---|---|---|
| Total[b] | 104.8 ± 5.8 | 149.8 ± 8.2 | < 0.001 | 1.4 |
| CD19[b] | 59.0 ± 4.5 | 86.5 ± 5.4 | < 0.001 | 1.5 |
| CD4[b] | 22.5 ± 1.1 | 20.3 ± 1.1 | NS | 0.9 |
| CD8[b] | 12.1 ± 0.5 | 12.7 ± 0.7 | NS | 1.0 |
| CD11c[b] | 2.2 ± 0.2 | 3.3 ± 0.3 | < 0.01 | 1.5 |

[a] Data are pooled from two experiments ($n = 13$), and statistical significance was determined by two-tailed Student's *t* test (NS: $p > 0.05$).

[b] Normal 4- to 5-wk-old B6 mice were implanted with 0.25 mg of E2 pellets or left untreated. At 8 wk of age, E2 pellets were removed and mice were immunized in the tail base with 5 $\mu$g of tAChR in CFA. Spleen cells were harvested 14 days later, and absolute number of cells was expressed as mean ± SEM per spleen ($\times 10^{-6}$).

Therefore, both enhanced expansion of B cells and production of type 1 cytokines by AChR-specific CD4 T cells occur in the spleen and correlate with the enhanced production of anti-tAChR IgG2a[b] and IgG2b isotypes in E2-pretreated mice.

We also examined whether estrogen administration affects the splenic APC populations and their capacity to secrete the Th1-promoting cytokine IL-12. Interestingly, we found that the E2-mediated up-regulation of IFN-$\gamma$ production by Ag-specific CD4$^+$ T cells was correlated with an increased capacity of splenic APCs, most likely DCs, to secrete IL-12 upon in vitro stimulation with SAC plus IFN-$\gamma$ (Fig. 6A). Within the multiple DC subsets, the lymphoid DC subtype characterized by expression of lineage-specific markers CD11c and CD8$\alpha$ (CD11c$^+$CD8$\alpha^+$CD11b$^-$) has been shown to produce higher amounts of IL-12 and to better trigger the development of Th1 cells as compared with CD11b$^+$ myeloid DCs (35, 36). In the present study, we observed that the frequency of CD8$\alpha^+$CD11c$^+$ DCs was higher in splenocytes from E2-treated mice than control mice (Fig. 6B, $p < 0.001$). In addition, we found that compared with control mice, spleens from E2-treated mice exhibited a 1.5- and 2-fold increase in the absolute number of splenic DCs and CD8$\alpha^+$ DCs, respectively (Table I and data not shown). Altogether, these data indicate that the E2-mediated increase in AChR-specific Th1 response is associated with an enhanced capacity of splenic APC to produce IL-12 through the recruitment of CD8$\alpha^+$ DC.

## Discussion

In the present study, we have analyzed whether exposure to estrogen modulates development of autoimmune myasthenia gravis through action on AChR-specific T and B cell responses. We show that E2 treatment enhances AChR-specific CD4$^+$ T cell expansion in vivo and their capacity to produce type 1 cytokines, such as IFN-$\gamma$ and TNF-$\alpha$, as well as IL-6, but not the Th2 cytokines IL-4, IL-5, and IL-10. These data are in agreement with our previous findings showing that E2 administration resulted in amplification of Ag-specific Th1 cell responses through estrogen receptor $\alpha$ (ER$\alpha$) expression in hemopoietic cells (37). Interestingly, we show in this study that the promoting effect of E2 on AChR-specific Th1 response was maintained in the absence of hormone supplementation during the development of the immune response. Thus, a sustained increase in serum E2 levels 3 wk before immunization was necessary and sufficient to enhance AChR-specific immunity. This exacerbation of AChR-specific Th1 responses mediated by E2 presensitization was lost in ER$\alpha$-deficient mice (our unpublished data). Using this protocol of time-limited exposure to E2, we further observed an increased severity of EAMG development associated with enhanced production of anti-tAChR Abs of IgG2a[b] and IgG2b isotypes. Such IgG subclasses have been shown previously to play an important role in susceptibility to EAMG in mouse (8, 34, 38, 39). Our data support the hypothesis that estrogens, as sex-specific factors, may be responsible for the higher susceptibility of women to MG through an enhancing effect on the development of autoimmune T and B cell responses to AChR.

Concerning the role of estrogens on polarization of T cell responses, it has been shown in vitro that, depending on the dose of hormone, E2 could modulate both pro- and anti-inflammatory activities of human T cell clones (40). Thus, it has been hypothesized that the response to estrogens might be biphasic with high levels driving a Th2-polarized cytokine secretion pattern and inhibiting autoimmunity, whereas low levels could promote Th1 immunity and susceptibility to cell-mediated autoimmune diseases (20). However, our data challenge this hypothesis and show that treatment with low to high doses of E2 increases AChR-specific Th1 cell response in vivo. Indeed, we provide evidence that over a wide dose range of E2 corresponding to estrus (0.1 mg of E2 pellet) to pregnancy levels (2.5 mg of E2 pellet) (27, 41), a strong enhancement of Ag-specific Th1 responses was observed. By contrast, type 2 cytokines were not up-regulated, demonstrating a selective increase in Th1 responsiveness. Our data contrast with previous experiments, showing that administration of similar doses of E2
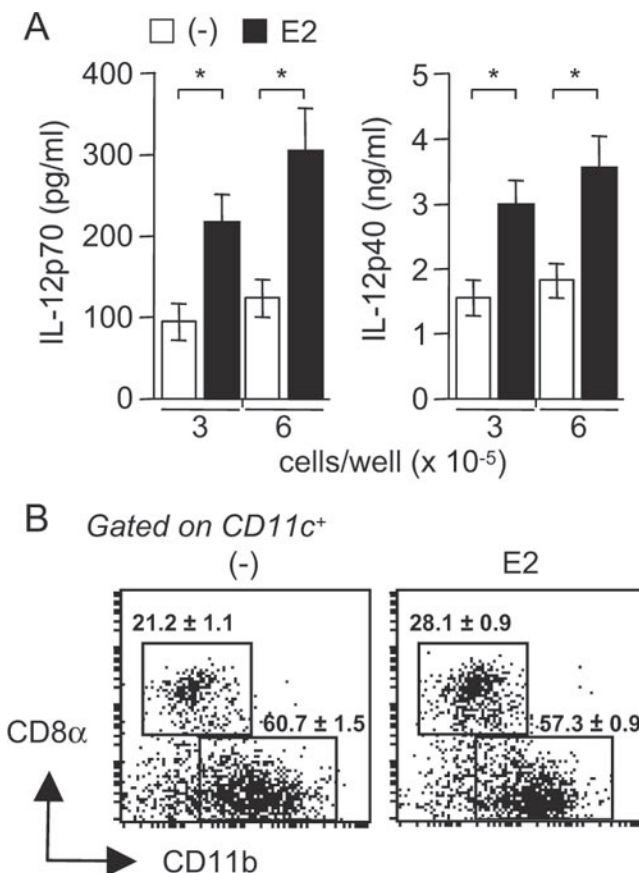


**FIGURE 6.** E2 enhances the capacity of splenic APC to produce IL-12 through the selective recruitment of CD8$\alpha^+$ DC. Normal 4- to 5-wk-old B6 mice were implanted with 0.25 mg of E2 pellets or left untreated. At 8 wk of age, E2 pellets were removed from E2-treated mice and immunization was performed as described in Fig. 5. Indicated numbers of spleen cells harvested 14 days after immunization were stimulated in 200 $\mu$l vol with SAC (1/5000 dilution) plus IFN-$\gamma$ (5 ng/ml) and IL-12 production was assessed by ELISA in 24-h culture supernatants (*A*). *B*, spleen cells were stained with indicated fluorescent Abs after collagenase IV digestion. DC subtypes were analyzed on $3 \times 10^4$ CD11c$^+$ viable cells as determined by propidium iodide exclusion. Data are from one representative experiment of three performed (*, $p < 0.05$).

(2.5–0.36 mg) 1 wk before immunization inhibits type 1 cytokine production and induces a modest increase in IL-10 production by Ag-specific CD4 T cells (27, 42). Together, these data emphasize the importance of the timing of E2 administration rather than the dose of hormone on T cell polarization. Indeed, we further demonstrated that E2 administration 3 wk before priming was required for the induction of enhanced Ag-specific Th1 cell responses. By contrast, starting hormonal treatment at the time of immunization had little, if any, effect on the establishment of T cell responses. This observation may also explain previous experiments showing that estrogen administration during the course of EAMG in rats had no effect on disease severity (43).

Th1 differentiation is driven by signaling pathways emanating from both TCR and IL-12R (44), and the involvement of IL-12 production by professional APCs such as DCs is essential for fixing, amplifying, and maintaining Th1 cell effector functions (45). Indeed, we showed that E2-mediated up-regulation of IFN-$\gamma$ production by Ag-specific T cells correlated with an increased capacity of splenic APCs to secrete IL-12 upon in vitro stimulation with SAC plus IFN-$\gamma$. Furthermore, the enhancement of Ag-specific Th1 response induced by E2 administration was completely abolished in IL-12R$\beta$2$^{-/-}$ mice (our unpublished data), demonstrating that the E2-mediated increase in Ag-specific Th1 cell development required a functional IL-12/IL-12R signaling pathway. Thus, it is likely that the higher propensity of E2-treated mice to mount Th1 cell responses in vivo could be due to the recruitment in situ of a phenotypically and functionally distinct population of DC. In support of this, it has been recently shown that estrogens were needed for the optimal development of DCs from bone marrow precursors in vitro (46). The requirement for estrogens during DC differentiation suggests a mechanism by which E2 levels in peripheral tissues might modulate both number and functional properties of DC in vivo, thereby influencing immune responses. Indeed, splenic DC numbers were increased in E2-treated mice, and this increase seems to affect preferentially CD8$\alpha^+$ DCs that have been shown to secrete higher amounts of IL-12 (35, 47). However, we cannot rule out that these quantitative and phenotypic changes in splenic DC populations might reflect secondary events due to an enhanced production of T cell-derived cytokines upon immunization of E2-treated mice, resulting in an increased recruitment and/or maturation of CD8$\alpha^+$ lymphoid DCs in T cell zones, as previously suggested (47).

Even though MG and EAMG are mediated by autoantibodies, CD4 T cells play a crucial role in the control of the autoimmune process. Using cytokine knockout mice, it has been shown that IFN-$\gamma$ and IL-12 were necessary for the development of EAMG, whereas IL-4 was dispensable (6–8). Although these data underline the importance of Th1 cytokines in the pathogenesis of this Ab-mediated autoimmune disease, it has also been shown that IL-5 and IL-10 could influence EAMG development (9–11). However, in our study, a role for such type 2 cytokines is unlikely because their production by AChR-specific CD4 T cells was not enhanced by E2 treatment. Thus, estrogens may contribute to the susceptibility to EAMG by promoting Th1 cell development in vivo and the subsequent development of pathogenic autoreactive B cells. Indeed, the induction of strongly Th1-polarized immune responses by IL-12 administration in vivo was found to provide optimal conditions for the development of EAMG both in mice (8, 38) and rats (3).

Interestingly, the enhanced CD4 T cell response measured in spleen from E2-presensitized AChR-immunized mice was also associated with an increase in cellularity that was mainly due to B cells. Such observation contrasts with previous studies showing decreased B cell lymphopoiesis in E2-treated mice (48). This effect was mainly observed in bone marrow of normal nonimmunized mice (48). Thus, the increased splenic B cell number reported in this work could be due to enhanced B cell proliferation induced by Ag-specific CD4 T cells. Furthermore, the drop in serum estrogen levels following E2 pellet removal could also increase B lymphopoiesis. Indeed, estrogen deficiency has been shown to stimulate B lymphocyte development in mice (49). Thus, in the spleen, polarized Th1 cells could provide help to AChR-specific B cells and support Ab class switching toward complement-fixing IgG2a$^b$ and IgG2b (6–8, 50). Because IL-6 is crucial for both the differentiation of activated B cells into plasma cells and the development of EAMG (51), the 3- to 4-fold increased serum level of these pathogenic IgG2a$^b$ and IgG2b isotypes found in E2-presensitized mice could be due to the increased production of this cytokine and could reflect a higher propensity to B cells to give rise to postswitch plasmocytes. A role for estrogens as trigger of immune complex-mediated autoimmune diseases has been well documented in murine lupus. It has been shown that E2 treatment of NZB/NZW F$_1$ lupus-prone mice accelerates Ab-mediated glomerulonephritis, resulting in earlier disease onset and increased mortality (21). Some molecular mechanisms for the estrogen-promoting effect on this autoimmune disease have recently been provided. E2 treatment of nonautoimmune mice transgenic for the H chain of an anti-DNA Ab led to the rescue from deletion of a population of autoreactive B cells due to enhanced Bcl2 expression (52–54). Likewise, E2 administration has been shown to increase plasma cell number and to enhance autoantibody production in B6 mice (55). According to these data, we could not exclude that in our study both enhanced survival of autoreactive B cells and increased Th1-driven AChR-specific Ab response are at work in the E2-mediated exacerbation of EAMG.

In this study, the reported deleterious effect of E2 treatment on EAMG underlines the complexity of the role of estrogens in autoimmunity. We and others have also demonstrated that estrogens could be associated with clinical improvement of cell-mediated autoimmune diseases such as EAE or collagen-induced arthritis (26, 27, 31). Using irradiation bone marrow chimeras, we have shown recently that the beneficial effect of E2 on EAE did not involve ER$\alpha$ signaling in hemopoietic cells. These data indicate that ER$\alpha$ expression in endothelial cells or in other tissues, such as CNS-resident microglia, may mediate the protective effect of E2 on EAE (31). Therefore, the ER$\alpha$-driven effects of E2 on hemopoietic vs nonhemopoietic tissues could in part explain the paradoxical effects of estrogens on Th1-associated autoimmune diseases. Our current hypothesis is that E2-mediated protection on autoimmunity could be dependent on the type of effector arms involved in immune responses. Because cell-mediated immune responses have been ultimately implicated in the pathogenesis of RA or MS, we hypothesize that E2 might dampen the inflammatory phase, leading to tissue injury in these autoimmune diseases by preventing inflammatory leukocyte recruitment in target organs. This phenomenon might be dependent on the maintenance of high plasma levels of E2 such as those found during late pregnancy. Reduction in estrogen level would result in disease reappearance or exacerbation, a situation that has been reported in MS and RA patients following delivery (19, 56–58). By contrast, estrogen would not be predicted to have protective effects in autoimmune diseases in which autoantibody production is central to much of tissue damage. This would explain why immune complex- or Ab-mediated autoimmune diseases such as SLE or MG, respectively, could worsen during pregnancy (20).

In conclusion, our study provides the first evidence that estrogens may contribute to the susceptibility to EAMG by promoting

AChR-specific Th1 cell expansion and the development of pathogenic autoreactive B cells. Understanding how E2 operates in modulating innate and adaptive immunity in vivo may provide new insights into the mechanisms by which sex-linked factors affect immunity and susceptibility to autoimmune diseases in women. In addition to its obvious relevance to early onset MG, these data could also have wide-ranging implications for the use of either estrogen or anti-estrogen therapies in other autoimmune diseases.

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## Disclosures

The authors have no financial conflict of interest.

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