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### Estrogen Signaling in Bystander Foxp3<sup>neg</sup> CD4<sup>+</sup> T Cells Suppresses Cognate Th17 Differentiation in *Trans* and Protects from Central Nervous System Autoimmunity **FREE**

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# Estrogen Signaling in Bystander Foxp3<sup>neg</sup> CD4<sup>+</sup> T Cells Suppresses Cognate Th17 Differentiation in *Trans* and Protects from Central Nervous System Autoimmunity

Laure Garnier,\* Sophie Laffont,\* Karine Lélou,\*<sup>1</sup> Nir Yogev,<sup>†,‡</sup> Ari Waisman,<sup>†</sup> and Jean-Charles Guéry\*

17β-Estradiol (E2) suppresses the development of experimental autoimmune encephalomyelitis (EAE) through estrogen receptor (ER) α, yet the cellular targets remain elusive. We have used an adoptive transfer model of myelin oligodendrocyte glycoprotein-specific CD4<sup>+</sup> T cells from 2D2 TCR transgenic mice. We show that in the recipient mice, ERα expression in bystander CD4<sup>+</sup> T cells, rather than in cognate 2D2 T cells, is required for the inhibition of Th17 cell differentiation by E2. Coadministration of estrogen-primed WT, but not ERα-deficient CD4<sup>+</sup> T cells, with naive 2D2 T cells lacking ERα inhibited the development of Th17 cell-mediated EAE. Suppression of Th17 cells and protection from EAE were maintained when ERα was deleted in Foxp3<sup>+</sup> regulatory T cells. We showed that in vivo PD-L1 blockade alleviated the anti-inflammatory action of E2 and that PD-1 expression on cognate but not bystander T cells was required for the E2-dependent inhibition of Th17 differentiation. In cotransfer experiments, we found that only WT but not PD-1<sup>KO</sup> 2D2 T cells were amenable to E2-dependent inhibition of Th17 differentiation. These results support the conclusion that the restriction of Th17 cell development by E2-primed bystander CD4<sup>+</sup> T cells requires cell-intrinsic PD-1 signaling within cognate T cells rather than induction of regulatory 2D2 T cells through PD-1 engagement. Altogether, our results indicate that pregnancy-level concentrations of estrogen signal in conventional Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells to limit the differentiation of cognate Th17 cells through a *trans*-acting mechanism of suppression that requires a functional PD-1/PD-L1 regulatory axis. *The Journal of Immunology*, 2018, 201: 3218–3228.

Multiple sclerosis is a T cell-mediated autoimmune disease with a female to male ratio of 3:1. Multiple sclerosis and its mouse model, experimental autoimmune encephalomyelitis (EAE), are characterized by the infiltration of inflammatory leukocytes, including autoreactive T cells, into the CNS, resulting in myelin damage (1). Reduced disease activity in women with multiple sclerosis is commonly observed during pregnancy, suggesting that sex hormones, such as estrogens, could downmodulate the autoimmune response and inflammation (2–4). Indeed, studies in EAE have clearly established protective effects of estrogen administration on disease activity (5–10). Likewise, protective effects of estrogens have been reported in

clinical trials using estril (11–13) or 17β-estradiol (E2) (14) in relapsing-remitting multiple sclerosis patients. These disease-modulating effects of exogenous estrogens in CNS autoimmunity are thought to account for the beneficial effects of pregnancy on clinical symptoms in women with multiple sclerosis (15, 16).

Estrogens bind to nuclear receptors, thereby directly or indirectly regulating the expression of numerous genes. Estrogens can also activate intracellular signaling pathways. There are two estrogen receptors (ERs): ERα and ERβ. These ERs, most notably ERα, are functionally expressed by many immune cells, including T cells (10), B cells (17), macrophages (18, 19), and dendritic cells (DCs) (20–24), suggesting that estrogens could regulate immunity through a direct effect on immunocompetent cells. Alternatively, ERs are expressed by CNS-resident cells such as neurons (25), astrocytes (25–27), and microglia (28). Recent works have shown that estrogens or ER-selective ligands can exert potent neuroprotective effects by acting in CNS-resident cells through ERα (25–27) or ERβ (28, 29). Thus, emerging evidence supports the notion that E2 could inhibit CNS autoimmunity and inflammation through distinct nonoverlapping mechanisms, namely anti-inflammatory and neuroprotective actions (15, 16).

In the active EAE model, priming of autoantigen-specific CD4 T cells in the secondary lymphoid tissues by DCs, which produce polarizing cytokines such as IL-12, IL-23, and IL-6, promotes the development of pathogenic CD4 T cells into Th1 and Th17 phenotype (30, 31). Both Th1 and Th17 cells can induce EAE, albeit with different symptoms, depending on the preferential recruitment of either cell type to the spinal cord or the brain parenchyma, respectively (32, 33). IL-23–driven GM-CSF secretion by pathogenic Th17 cells is essential for the induction of neuroinflammation in EAE (34, 35). A consistent observation in E2-mediated EAE protection is the strong anti-inflammatory effect characterized by the inhibition of autoantigen-specific CD4<sup>+</sup> T cell responses in

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Abbreviations used in this article: DC, dendritic cell; E2, 17β-estradiol; EAE, experimental autoimmune encephalomyelitis; ER, estrogen receptor; LNC, lymph node cell; MOG, myelin oligodendrocyte glycoprotein; Treg, regulatory T.

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lymphoid organs, including both Th1 (5, 7, 8, 10, 36) and Th17 cells (9, 10). This immunosuppressive mechanism triggered by E2 treatment occurs early in the course of the myelin oligodendrocyte glycoprotein (MOG)-specific immune response in draining lymph nodes (10) and is likely to account for the sustained suppression of EAE generally observed in E2-treated mice (5, 7, 36).

Several hypotheses have been proposed to explain the anti-inflammatory effect of E2 on EAE, such as enhanced expansion of Foxp3<sup>+</sup> regulatory T (Treg) cells (9, 37), induction of tolerogenic DCs (38) or regulatory B cells (39), and direct inhibition of Th17 cells (40). However, the precise dominant cellular targets in which estrogen signals *in vivo* to inhibit Th17 cells priming in draining lymph nodes had remained elusive until we established, using a conditional knockout strategy, that ER $\alpha$  expression in T cells, but not DCs nor macrophages, was necessary and sufficient to mediate the protective effect of E2 on EAE (10).

In the current study, we seek to precisely identify the T cell populations targeted by E2 through the generation of new mouse models in which ER $\alpha$  was selectively invalidated in cognate MOG-specific CD4<sup>+</sup> T cells or bystander CD4<sup>+</sup> T cells, including Foxp3<sup>+</sup> Treg cells. Altogether, our findings identified bystander Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells as the main cellular targets of E2 that restrict Th17 cell differentiation *in trans* and protect from CNS autoimmunity through a mechanism that requires PD-1 on cognate CD4<sup>+</sup> T cells but not Treg cells.

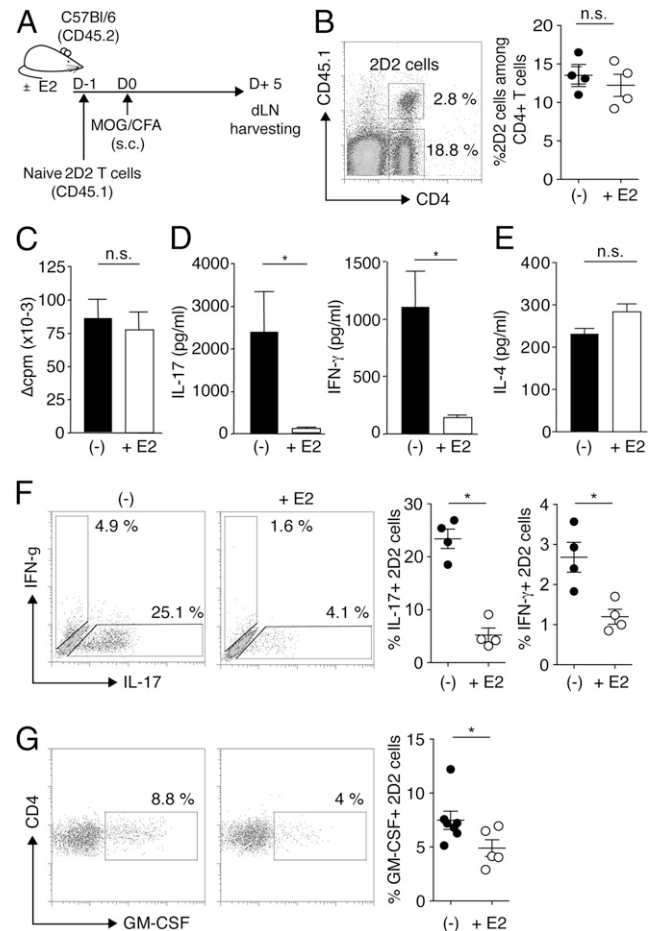
## Materials and Methods

### Mice

Female C57BL/6J (B6) mice were purchased from the Centre d'Élevage R. Janvier (Le Genest St. Isle, France). Mice with a disrupted *Esr1* gene (hereafter called ER $\alpha$ <sup>KO</sup>), previously described (41), were backcrossed onto the B6 background for at least 10 generations. To generate mice lacking T and B cells and deficient for ER $\alpha$ , B6, and ER $\alpha$ <sup>KO</sup>, mice were crossed to B6 Rag2<sup>KO</sup> mice. To generate TCR transgenic mice lacking ER $\alpha$ , B6 ER $\alpha$ <sup>KO</sup> mice were crossed to OVA-specific OT-2 CD4.1 mice or 2D2 CD45.1 mice (42) expressing a MOG<sub>35–55</sub>/I-A<sup>b</sup>-specific TCR. Mice selectively lacking ER $\alpha$  in T lymphocytes or in DCs have been described elsewhere (10). They were obtained by crossing ER $\alpha$ <sup>fl/fl</sup> mice (41) with mice expressing the Cre recombinase under the control of CD4 (43) or CD11c (44) promoter, respectively. The targeted exon 2 of the *Esr1* gene encodes the first zinc finger of the DNA-binding domain of ER $\alpha$ . Cre-mediated recombination of the conditional floxed allele leads to unambiguous inactivation of ER $\alpha$ . In the current study, we generated mice selectively lacking ER $\alpha$  in Treg cells (Foxp3<sup>+</sup> cells) (Foxp3<sup>YFPcre</sup> ER $\alpha$ <sup>fl/fl</sup>) by crossing ER $\alpha$ <sup>fl/fl</sup> mice with mice expressing the Cre recombinase and the YFP protein fluorescent genes under the control of Foxp3 promoter (45). PD-1<sup>KO</sup> (46) and 2D2 PD-1<sup>KO</sup> (47) mice have been previously described. For the generation of irradiation bone marrow chimeras, mice were  $\gamma$ -irradiated (8.5 Gy [850 rad], [<sup>137</sup>Cs] source) the day before reconstitution with  $5 \times 10^6$  PD-1<sup>KO</sup> or WT bone marrow cells per mouse. Mice were used 8 wk after reconstitution. All mice were housed in specific pathogen-free conditions. All animals were handled according to the Animal Care and Use of Laboratory Animal guidelines of the French Ministry of Research (study approval number 05187.01).

### Induction of EAE

Purified CD4<sup>+</sup> T cells ( $4\text{--}5 \times 10^6$  cells) from CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> Foxp3<sup>YFPcre</sup> ER $\alpha$ <sup>fl/fl</sup>, and their littermate control mice (ER $\alpha$ <sup>fl/fl</sup> or Foxp3<sup>YFPcre</sup> ER $\alpha$ <sup>+/+</sup>) pretreated or not with E2, were transferred *i.v.* together with isolated naive 2D2 T cells ( $2 \times 10^4$  cells) into castrated RAG<sup>KO</sup> ER $\alpha$ <sup>KO</sup> mice (male or female). One day after adoptive transfer, recipient mice were immunized *s.c.* in the flanks with 50  $\mu$ g of MOG<sub>35–55</sub> peptide (Neosystem, Strasbourg, France) emulsified in IFA supplemented with *Mycobacterium tuberculosis* (H37RA; BD, St. Louis, MO) (CFA at 1 mg/ml) and were injected *i.p.* with 200 ng of pertussis toxin (Calbiochem, Darmstadt, Germany) on day 0. Mice receiving CD4<sup>+</sup> T cells pretreated with E2 were also treated with E2 to maintain E2 signals all along the experiment. Mice were daily scored for clinical signs of disease as follows: 0, no detectable signs of EAE; 1, complete limp tail; 2, limp tail and hindlimb weakness; 3, severe hindlimb

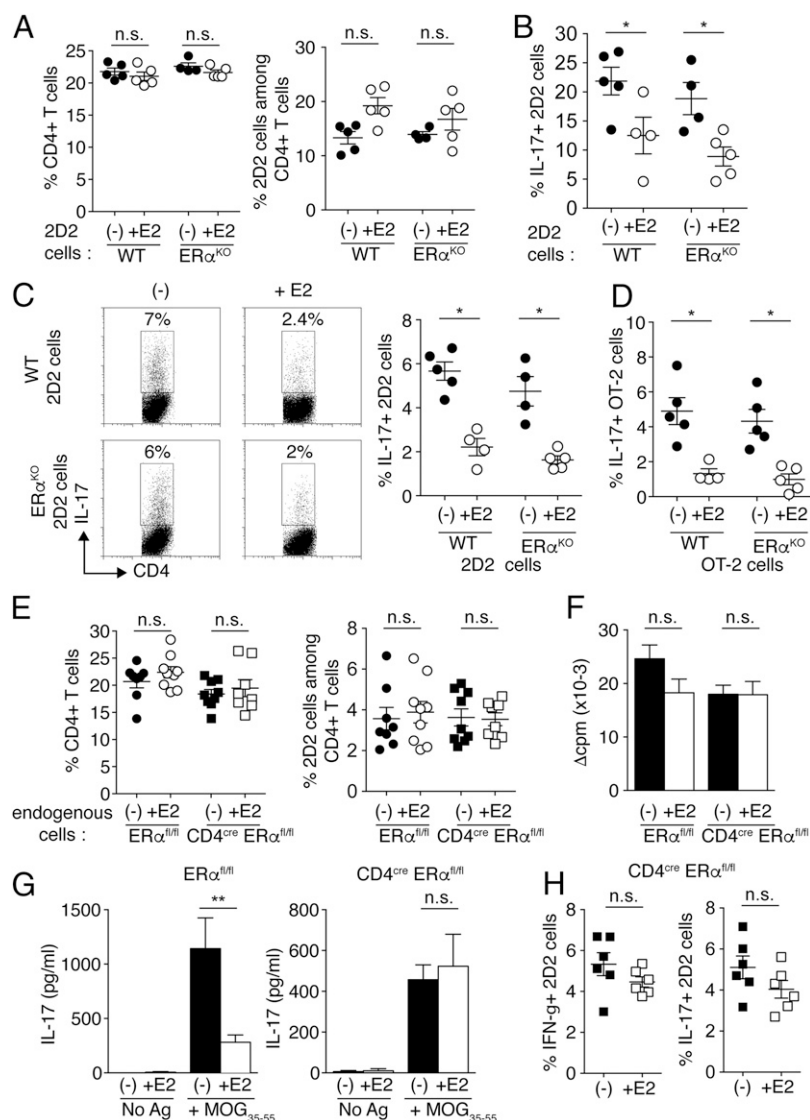


**FIGURE 1.** E2 treatment inhibits the development of MOG-specific Th1 and Th17 2D2 CD4<sup>+</sup> T cells *in vivo*. (A) B6 mice were implanted with E2 pellet 7 d before immunization with MOG<sub>35–55</sub> in CFA and adoptively transferred with naive 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells 1 d before immunization. Control untreated mice were sham operated and received no pellet. (B) At day 5, the frequency of 2D2 CD45.1<sup>+</sup> T cells was analyzed by flow cytometry. Error bars show mean  $\pm$  SEM. (C–E) LNC were restimulated with 10  $\mu$ M MOG<sub>35–55</sub> peptide. (C) Proliferation was quantified after 48-h culture. Error bars show mean  $\pm$  SEM.  $n = 4$  mice per group. (D) IL-17 and IFN- $\gamma$  secretion in 48-h culture supernatants were quantified by ELISA. Error bars show mean  $\pm$  SEM.  $n = 4$  mice per group,  $*p < 0.05$ , Mann–Whitney  $U$  test. (E) IL-4 secretion in 48-h culture supernatants was quantified by ELISA. Error bars show mean  $\pm$  SEM.  $n = 4$  mice per group. (F) IL-17 and IFN- $\gamma$  intracellular production in 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells was assessed by flow cytometry after PMA/ionomycin stimulation. Error bars show mean  $\pm$  SEM.  $n = 4$  mice per group,  $*p < 0.05$ , Mann–Whitney  $U$  test. (G) GM-CSF intracellular production in 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells was assessed by flow cytometry after PMA/ionomycin stimulation. Error bars show mean  $\pm$  SEM.  $n = 4$  mice per group,  $*p < 0.05$ , Mann–Whitney  $U$  test. n.s., not significant.

weakness; 4, complete bilateral hindlimb paralysis; 5, complete hindlimb paralysis and forelimb weakness; 5.5, total paralysis of both forelimbs and hindlimbs; and 6, death. At the peak of disease, mice were sacrificed, and mononuclear cells were isolated from the spinal cord or brain, counted, and analyzed for cytokine expression. Cumulative disease indexes were calculated as the sum of daily clinical score of each individual mouse.

### E2 treatment

For E2 treatment, we used the standard protocol described elsewhere (10). Briefly, female mice were implanted *s.c.* in the scapular region with an E2 pellet (2.5 mg, 60-d release; Innovative Research of America) 7 d before CD4<sup>+</sup> T cells purification or immunization with MOG<sub>35–55</sub> peptide according to the experiments. Control untreated mice were sham operated and received no pellet.



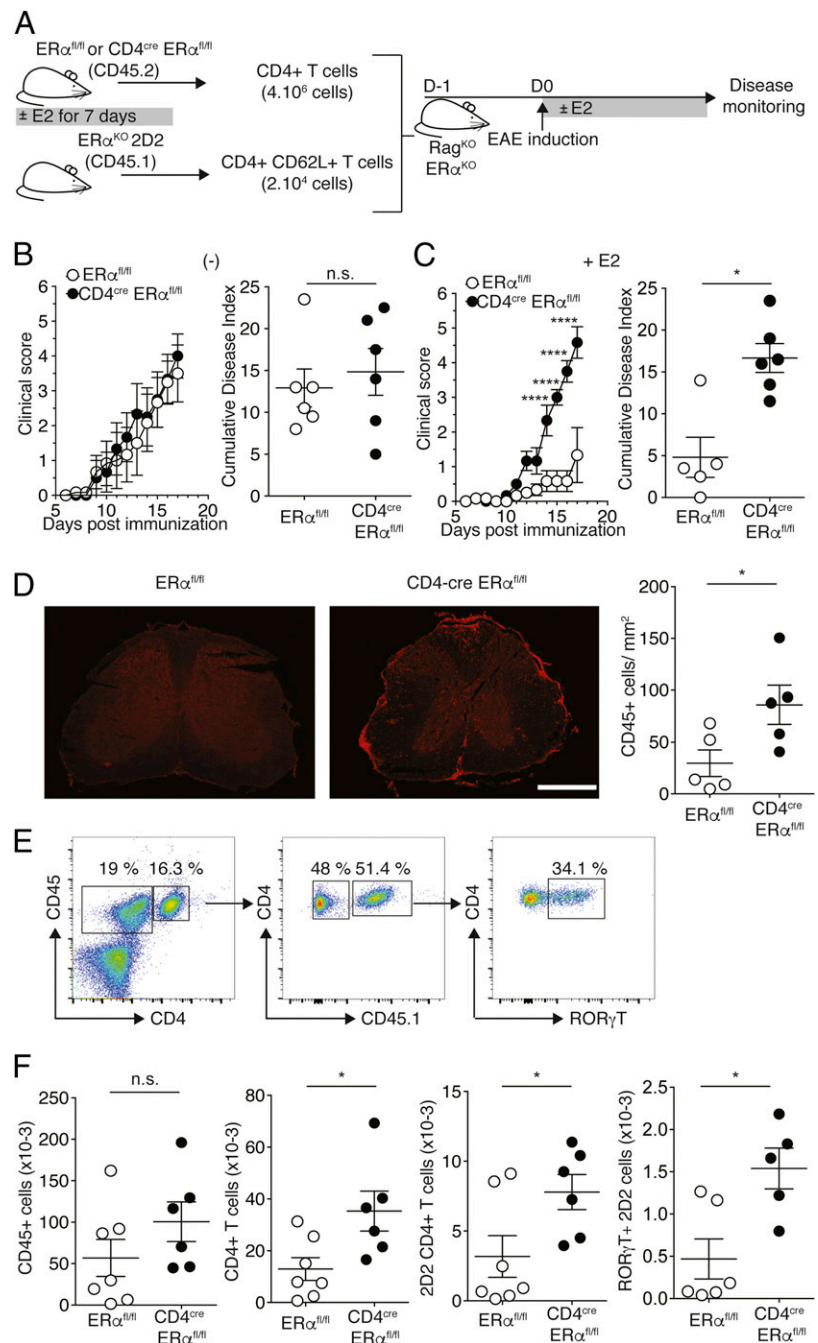
**FIGURE 2.** ERα signaling in endogenous but not in cognate CD4<sup>+</sup> T cells is required for E2-mediated inhibition of Th17 cell development in vivo. (**A–H**) B6 mice were treated or not with E2 pellets as in Fig. 1 7 d before immunization with MOG<sub>35–55</sub> in CFA. One day before immunization, mice were adoptively transferred with WT or ERα<sup>KO</sup> 2D2 naive CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. (**A**) The frequencies of CD4<sup>+</sup> T cells and 2D2 CD45.1<sup>+</sup> were assessed at day 5. Error bars show mean ± SEM. (**B**) IL-17 intracellular production in PMA/ionomycin-stimulated 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. Error bars show mean ± SEM, \**p* < 0.05, Mann–Whitney *U* test. (**C**) IL-17 intracellular production in MOG<sub>35–55</sub>-stimulated 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells analyzed by flow cytometry. Error bars show mean ± SEM, \**p* < 0.05, Mann–Whitney *U* test. (**D**) B6 mice were implanted with E2 pellet 7 d before immunization with OVA<sub>323–339</sub> in CFA. Control or E2-treated mice were adoptively transferred with WT or ERα<sup>KO</sup> OT-2 naive CD45.1<sup>+</sup> CD4<sup>+</sup> T cells and immunized with OVA<sub>323–339</sub> in CFA. IL-17 intracellular production of OT-2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells was analyzed as in (**B**). Error bars show mean ± SEM, \**p* < 0.05, Mann–Whitney *U* test. (**E–H**) ERα<sup>fl/fl</sup> and CD4<sup>Cre</sup> ERα<sup>fl/fl</sup> were implanted or not with E2 pellet 7 d before immunization with MOG<sub>35–55</sub> in CFA. One day before immunization, mice were adoptively transferred with naive 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. (**E**) Frequencies of CD4<sup>+</sup> T cells and 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells were assessed at day 5. Error bars show mean ± SEM. This graph represents a pool of two independent experiments. (**F**) Cell proliferation was assessed after LNC restimulation with MOG<sub>35–55</sub> peptide. Error bars show mean ± SEM. This graph represents a pool of two independent experiments, *n* = 9 or 10 mice per group. (**G**) LNC were restimulated or not with MOG<sub>35–55</sub> peptide, and IL-17 secretion in 72-h culture supernatant was quantified by ELISA. This graph represents a pool of two independent experiments, *n* = 9 or 10 mice per group. Error bars show mean ± SEM, \*\**p* < 0.01, Mann–Whitney *U* test. (**H**) IFN-γ and IL-17 intracellular production was assessed in 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells upon PMA/ionomycin stimulation by flow cytometry. Error bars show mean ± SEM. n.s., not significant.

### T cell assays

Naive 2D2 T cells ( $1\text{--}2 \times 10^6$  or  $0.15 \times 10^6$  cells) either purified from WT or ERα<sup>KO</sup> mice were injected into WT, CD4<sup>Cre</sup> ERα<sup>fl/fl</sup>, CD11c<sup>Cre</sup> ERα<sup>fl/fl</sup>, or Foxp3<sup>YFP</sup> ERα<sup>fl/fl</sup> recipient mice pretreated or not with E2. The following day, recipient mice were immunized in the flank with MOG<sub>35–55</sub>/CFA as for EAE induction. Five to six days postimmunization, draining lymph nodes were harvested. For the recall assay with lymph node cells (LNC), total LNC ( $0.5 \times 10^6$  cells) were in vitro stimulated with MOG<sub>35–55</sub> peptide in HL-1 synthetic medium. Cultures were incubated for 72 h in a humidified atmosphere of 5% CO<sub>2</sub> in air. For T cell proliferation assays, cells were pulsed during the last 8 h of culture

with 1 μCi of [<sup>3</sup>H]TdR (GE Healthcare, Little Chalfont, U.K.). Cells were harvested onto glass fiber filter membranes, and [<sup>3</sup>H]TdR incorporation was measured by a MicroBeta TriLux luminescence counter (PerkinElmer, Waltham, MA). For cytokine analysis, LNC were in vitro stimulated with 10 μM MOG<sub>35–55</sub> peptide in HL-1 synthetic medium, and cytokine concentrations were measured in 72-h culture supernatant by ELISA using specific mAb pairs from BD Pharmingen. LNC ( $3 \times 10^6$  cells) were also ex vivo stimulated for 5 h with PMA/ionomycin (50 and 500 ng/ml, respectively) in presence of brefeldin A for the last 2 h. IL-17, IFN-γ, and GM-CSF intracellular production of 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells was then assessed by flow cytometry.

**FIGURE 3.** E2-mediated ER $\alpha$  signaling within endogenous CD4<sup>+</sup> T cells is necessary and sufficient to delay EAE disease onset. **(A)** Experiment model: Immunodeficient ER $\alpha^{\text{KO}}$  Rag $\alpha^{\text{KO}}$  mice were transferred with naive ER $\alpha^{\text{KO}}$  2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells together with CD4<sup>+</sup> T cells purified either from CD4<sup>Cre</sup> ER $\alpha^{\text{fl/fl}}$  or control ER $\alpha^{\text{fl/fl}}$  mice that have been treated or not with E2 for 7 d as in Fig. 1. E2 treatment was maintained in recipient mice. After T cell transfer, mice were immunized with MOG<sub>35–55</sub> in CFA to induce EAE. **(B)** Untreated sham-operated control mice were daily monitored for clinical signs of EAE, and the cumulative disease index (CDI) was calculated for each mouse. Error bars show mean  $\pm$  SEM. **(C)** E2-treated mice were daily monitored for clinical signs of EAE, and the CDI was calculated for each mouse. Error bars show mean  $\pm$  SEM, statistical significance of differences between groups for EAE clinical signs was determined using the two-way ANOVA test, \*\*\*\* $p < 0.0001$ . Statistical significance of differences between groups for CDI, \* $p < 0.05$ , Mann–Whitney  $U$  test. **(D–F)** On day 17 to 18 post-disease induction, spinal cords (SC) were harvested. **(D)** Confocal microscopy showing CD45 staining on SC section from mice transferred with CD4<sup>+</sup> T cells purified from ER $\alpha^{\text{fl/fl}}$  or CD4<sup>Cre</sup> ER $\alpha^{\text{fl/fl}}$  mice treated with E2 (scale bar, 500  $\mu\text{m}$ ). Error bars show mean  $\pm$  SEM, \* $p < 0.05$ , Mann–Whitney  $U$  test. **(E)** Flow cytometry staining representing CD45<sup>+</sup>, CD4<sup>+</sup> CD45.1<sup>+</sup>, and CD45.1<sup>+</sup> ROR $\gamma\text{T}$ <sup>+</sup> cells within SC-infiltrating cells. **(F)** Absolute numbers of CD45<sup>+</sup> cells, total CD4<sup>+</sup> T cells, 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells, and ROR $\gamma\text{T}$ -expressing 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells infiltrating the SC were calculated. Error bars show mean  $\pm$  SEM, \* $p < 0.05$ , Mann–Whitney  $U$  test. n.s., not significant.



### Isolation of CNS-infiltrating cells

On the indicated day postimmunization, mice were anesthetized and perfused intracardially with ice-cold PBS. Brain and spinal cord were harvested, grinded, and digested for 40 min with collagenase D (1 mg/ml) (Roche, Indianapolis, IN) and DNase I (0.2 mg/ml) (Sigma) in HBSS containing 20 mM hepes under continuous agitation at 37°C. Cells were then separated by centrifugation (1000 rpm for 40 min, 18°C) on a discontinuous isotonic Percoll gradient containing 70 and 37% layers. Mononuclear cells at the 70–37% interface were collected and washed extensively in FACS buffer containing 1% FCS, 2 mM EDTA, and 0.1% NaN<sub>3</sub> in PBS.

### Statistical analysis

For comparison of EAE clinical scores between groups, repeated measures two-way ANOVA was performed followed by a Bonferroni post hoc test by using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Otherwise, pairwise comparisons between groups were conducted either using the Mann–Whitney  $U$  test or one-way ANOVA test. All graphs show mean and SEM. A  $p$  value  $< 0.05$  was considered significant. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

### Results

#### E2 treatment inhibits the development of IFN- $\gamma$ -, IL-17-, and GM-CSF-producing MOG-specific 2D2 CD4<sup>+</sup> T cells in draining lymph nodes

To directly assess the inhibitory effect of E2 on CD4<sup>+</sup> T cell priming and differentiation in vivo, we set up an adoptive transfer model in which naive MOG-specific 2D2 Ly5.1<sup>+</sup> CD4<sup>+</sup> T cells were i.v. injected into Ly5.2 B6 mice treated or not with E2 6 d before. Mice were then immunized with MOG peptide in CFA, and draining lymph nodes were collected 5 d later (Fig. 1A). As shown in Fig. 1B, 2D2 CD4<sup>+</sup> T cells were readily detected in LN cells from both control and E2-treated mice with similar frequency and absolute numbers. These data suggested that T cell homing to inflamed LN as well as expansion of MOG-specific T cells were not impaired by E2 treatment. Accordingly, the recall proliferative response of 2D2 CD4<sup>+</sup> T cells induced by MOG<sub>35–55</sub> in vitro was

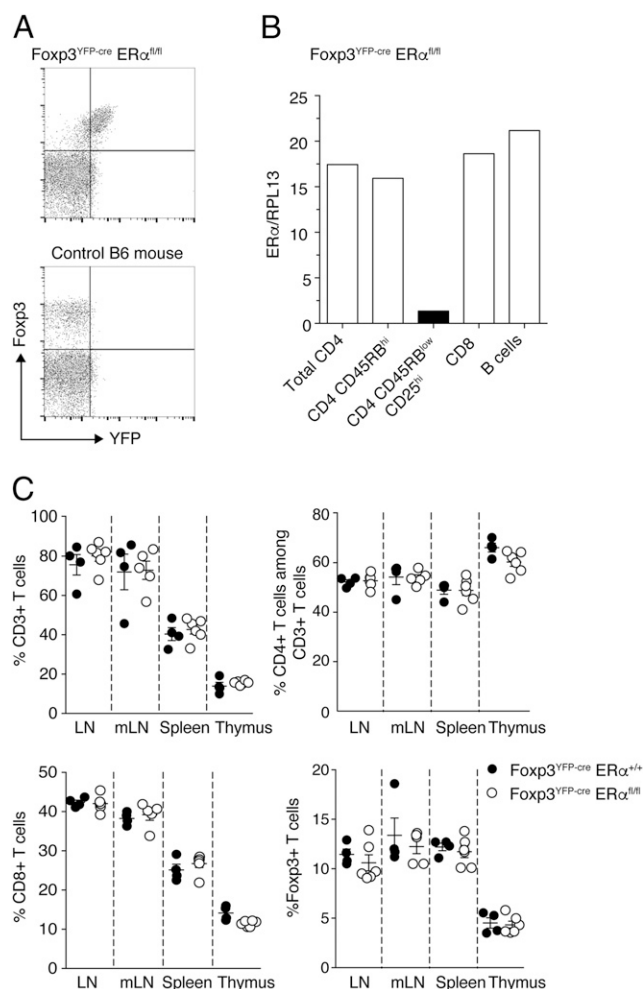


similar between both groups (Fig. 1C). Lack of effect of E2 treatment on recruitment and proliferation of Ag-specific T cells in LN strikingly contrasted with the strong inhibitory effect of E2 treatment on the development of MOG-specific 2D2 effector Th cells, producing either IL-17 or IFN- $\gamma$  (Fig. 1D). Unlike inflammatory cytokines, IL-4 production by MOG-specific 2D2 CD4<sup>+</sup> T cells was not altered by E2 treatment (Fig. 1E). In agreement with these results, the frequency of 2D2 CD4<sup>+</sup> T cells producing either IL-17 or IFN- $\gamma$  was strongly reduced in E2-treated mice (Fig. 1F). Likewise, E2 treatment also inhibited the development of GM-CSF-producing 2D2 CD4<sup>+</sup> T cells (Fig. 1G). Thus, raising serum E2 concentrations in recipient mice can restrict the differentiation of adoptively transferred naive T cells into effector Th1 and Th17, including GM-CSF-producing T cells, while sparing Th2 development.

*E2 inhibits early MOG-specific Th1/Th17 cell development in draining lymph nodes through ER $\alpha$  signaling in bystander but not cognate CD4<sup>+</sup> T cells*

We first investigated whether the E2 inhibitory effect was mediated through ER $\alpha$  signaling in cognate CD4<sup>+</sup> T cells. To address this issue, we generated ER $\alpha$ -deficient Ly5.1 2D2 TCR transgenic mice from which we purified naive CD4<sup>+</sup> T cells that were used in the adoptive transfer model depicted in Fig. 1A. After priming with MOG in CFA, similar frequency and numbers of ER $\alpha$ <sup>+/+</sup> and ER $\alpha$ <sup>KO</sup> 2D2 CD4<sup>+</sup> T cells were recovered from the draining lymph nodes of B6 recipient mice whether they were treated or not with E2 (Fig. 2A). In E2-treated mice, the inhibition of IL-17<sup>+</sup> 2D2 T cell development was not dependent on ER $\alpha$  expression in cognate 2D2 T cells (Fig. 2B, 2C), whether 2D2 cells were restimulated ex vivo with PMA/ionomycin (Fig. 2B) or their cognate peptide Ag (Fig. 2C). Similar adoptive transfer experiments were performed using naive OVA-specific OT-2 CD4<sup>+</sup> T cells expressing or not expressing ER $\alpha$  (Fig. 2D). As for 2D2 CD4<sup>+</sup> T cells, WT or ER $\alpha$ <sup>KO</sup> IL-17<sup>+</sup> OT-2 CD4<sup>+</sup> T cells developed at a similar frequency in control untreated mice, indicating that at steady-state, ER $\alpha$  signaling had no major impact on Th17 development in this experimental setting (Fig. 2D). Again, treatment of recipient mice with E2 strongly inhibited the development of IL-17<sup>+</sup> OT-2 cells irrespective of ER $\alpha$  expression in these cells (Fig. 2D). Altogether, these data show that E2/ER $\alpha$  signaling in cognate CD4<sup>+</sup> T cells is dispensable for the inhibition of Th17 cell differentiation.

We next examined the respective contribution of ER $\alpha$  signaling in recipient T lymphocytes or DCs in the E2-mediated inhibition of 2D2 Th1/Th17 cell priming using conditional ER $\alpha$  KO mice lacking ER $\alpha$  expression in T lymphocytes (CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup>) or in the DC lineage (CD11c<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup>). In agreement with our previous work showing that E2 could efficiently prevent EAE in mice lacking ER $\alpha$  in the DC lineage (10), we found that the priming of adoptively transferred 2D2 CD4<sup>+</sup> T cells into IFN- $\gamma$ -, IL-17-, and GM-CSF-producing CD4<sup>+</sup> T cells was still inhibited by E2 in the CD11c<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> mice as efficiently as in WT mice (Supplemental Fig. 1A, 1B). In striking contrast to the CD11c<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> mice, the inhibitory effect of E2 on 2D2 Th1/Th17 cell priming was lost in CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> mice lacking ER $\alpha$  expression in T lymphocytes (Fig. 2G, 2H). In agreement with previous data, the frequency of 2D2 CD4<sup>+</sup> T cells that expanded in draining lymph nodes were similar between ER $\alpha$ <sup>fl/fl</sup> or CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> recipient mice whether they were treated or not with E2 (Fig. 2E). Whereas the recall proliferative response of 2D2 CD4<sup>+</sup> T cells stimulated with MOG peptide in vitro was also similar between all groups (Fig. 2F), IL-17 production was strongly reduced in E2-treated ER $\alpha$ <sup>fl/fl</sup> control mice (Fig. 2G). This inhibitory effect of E2 was lost in CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> recipient mice (Fig. 2G). Direct analysis of the frequency of IFN- $\gamma$ - or IL-17-producing 2D2 CD4<sup>+</sup> T cells



**FIGURE 4.** Characterization of Foxp3<sup>YFP-Cre</sup>/ER $\alpha$ <sup>fl/fl</sup> mice. (A) Foxp3<sup>+</sup> cells from Foxp3<sup>Yfp-Cre</sup>/Yfp-Cre ER $\alpha$ <sup>fl/fl</sup> female mice express YFP. (B) Naive CD4<sup>+</sup> T cells, CD4<sup>+</sup> Treg cells, CD8<sup>+</sup> T cells, and B cells were sorted from Foxp3<sup>Yfp-Cre</sup>/Yfp-Cre ER $\alpha$ <sup>fl/fl</sup> mice by flow cytometry and analyzed for genomic ER $\alpha$  expression. (C) Frequency of T cells (CD3<sup>+</sup>), CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and Treg cells (Foxp3<sup>+</sup> YFP<sup>+</sup>) were assessed in lymph nodes, mesenteric lymph nodes, spleen, and thymus of Foxp3<sup>Yfp-Cre</sup>/Yfp-Cre ER $\alpha$ <sup>fl/fl</sup> mice and their littermate ER $\alpha$ <sup>fl/fl</sup> control mice.

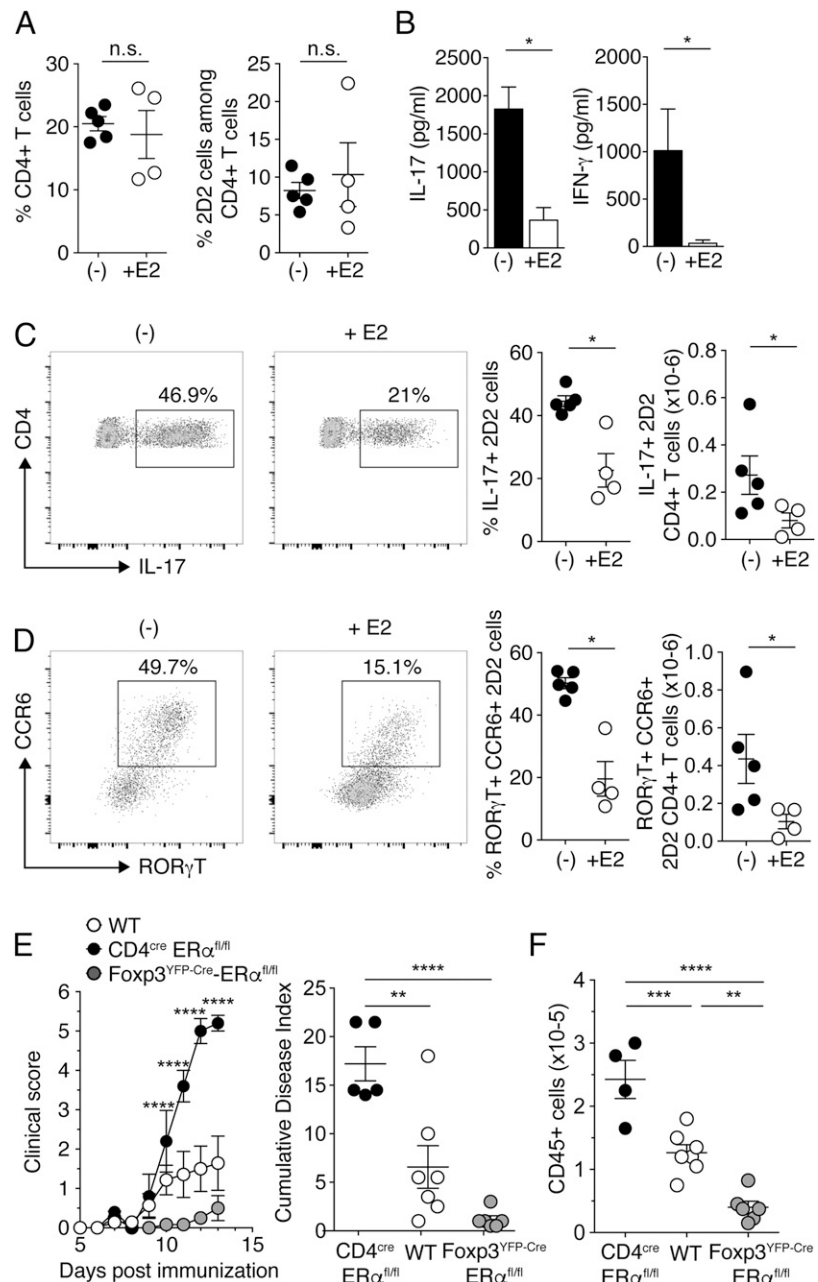
confirmed the absence of E2-mediated inhibition in recipient mice lacking ER $\alpha$  in T cells (Fig. 2H, Supplemental Fig. 2).

Among T cells, *Esr1* mRNA transcripts were expressed at higher level in CD4<sup>+</sup> T cell as compared with CD8<sup>+</sup> T cells. In CD4<sup>+</sup> T cells, *Esr1* gene expression was similar between CD45RB<sup>hi</sup> CD25<sup>+</sup> cells and CD45RB<sup>low</sup> CD25<sup>+</sup> cells (Supplemental Fig. 1C). As expected, ER $\alpha$  transcripts were undetectable in T lymphocytes purified from CD4<sup>Cre</sup> ER $\alpha$ <sup>fl/fl</sup> mice (Supplemental Fig. 1C). Inhibition of 2D2 T cell priming into IL-17- and IFN- $\gamma$ -producing cells still persisted in CD8<sup>+</sup> T cell-depleted mice, indicating that CD8<sup>+</sup> T cells are dispensable for the suppressive effect of E2 on MOG-specific Th1/Th17 differentiation (Supplemental Fig. 1D). Altogether, our results indicated that E2 inhibits early Th1/Th17 cell development in draining lymph nodes through a *trans*-acting mechanism of suppression, which required ER $\alpha$  signaling in the host CD4<sup>+</sup> T cell compartment but not in the cognate MOG-specific 2D2 CD4<sup>+</sup> T cell population.

*ER $\alpha$  signaling in bystander CD4<sup>+</sup> T cells inhibits MOG-specific Th17 cell development and protects from EAE*

To directly demonstrate that ER $\alpha$  expression within endogenous host CD4<sup>+</sup> T cells was necessary and sufficient for the E2-mediated

**FIGURE 5.** ER $\alpha$  expression in bystander Treg cells is dispensable for suppression of 2D2 Th17 cell development and EAE development. (**A–D**) Foxp3<sup>Yfp-Cre/Yfp-Cre</sup> ER $\alpha^{fl/fl}$  mice were implanted or not with E2 pellet as in Fig. 1. Seven days before immunization with MOG<sub>35–55</sub> in CFA. One day before immunization, mice were adoptively transferred with WT naive 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. (A) At day 6, the frequency of CD4<sup>+</sup> T cells and the frequency of 2D2 CD4<sup>+</sup> T cells among total CD4<sup>+</sup> T cells were assessed. Error bars show mean  $\pm$  SEM. (B) IL-17 and IFN- $\gamma$  secretion in 72-h culture supernatant were quantified by ELISA after LNC restimulation with MOG<sub>35–55</sub> peptide. Error bars show mean  $\pm$  SEM,  $n = 4$  or 5 mice per group. \* $p < 0.05$ , Mann–Whitney  $U$  test. (C) Frequency and absolute number of IL-17<sup>+</sup> 2D2 T cells were assessed in dLN after PMA/ionomycin stimulation by flow cytometry. Error bars show mean  $\pm$  SEM, \* $p < 0.05$ , Mann–Whitney  $U$  test. (D) Frequency and absolute number of CCR6<sup>+</sup> ROR $\gamma$ T<sup>+</sup> 2D2 T cells in dLN. Error bars show mean  $\pm$  SEM, \* $p < 0.05$ , Mann–Whitney  $U$  test. (**E**) Disease curves and CDI of mice transferred with naive 2D2 CD45.1<sup>+</sup> T cells together with CD4<sup>+</sup> T cells purified from either ER $\alpha^{fl/fl}$ , CD4<sup>Cre</sup> ER $\alpha^{fl/fl}$ , or Foxp3<sup>Yfp-Cre/Yfp-Cre</sup> ER $\alpha^{fl/fl}$  mice and immunized with MOG<sub>35–55</sub> in CFA as in Fig. 4. Error bars show mean  $\pm$  SEM, statistical significance of differences between groups for EAE clinical signs was determined using two-way ANOVA test, \*\*\*\* $p < 0.0001$ . Statistical significance of differences between groups for CDI, \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , one-way ANOVA test. (**F**) Hematopoietic CD45<sup>+</sup> cells infiltration in spinal cord of mice at day 13 postimmunization. Error bars show mean  $\pm$  SEM, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , one-way ANOVA test. dLN, draining lymph node; n.s., not significant.

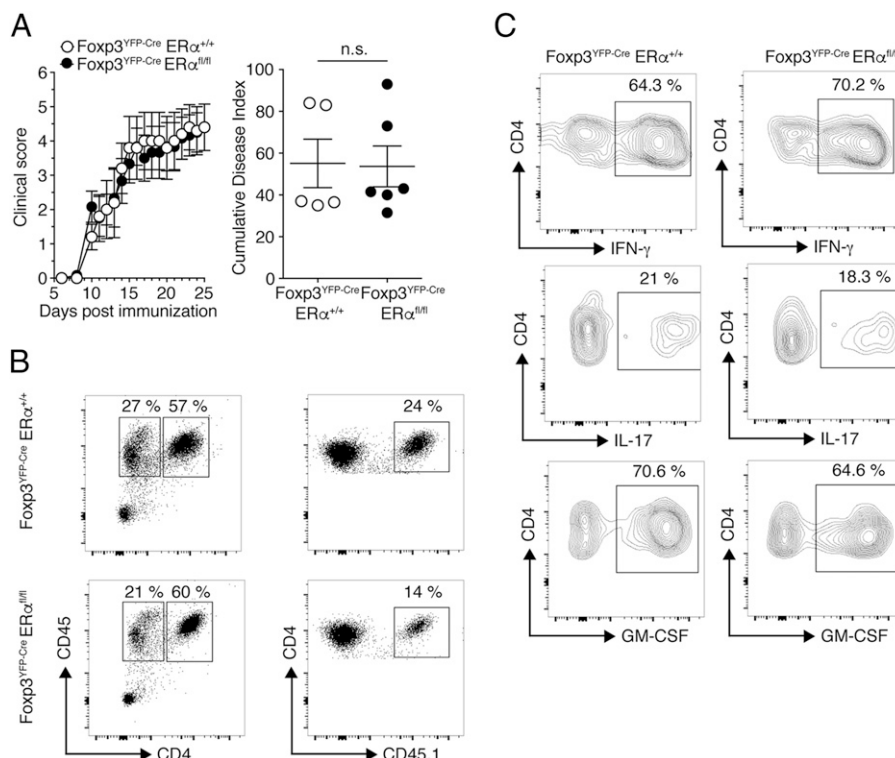


protection of EAE, we set up an experimental model in which ER $\alpha^{KO}$  Rag2<sup>KO</sup> mice were replenished with CD4<sup>+</sup> T cells purified from either WT or CD4<sup>Cre</sup> ER $\alpha^{fl/fl}$  C57BL/6 mice together with low numbers of naive ER $\alpha^{-/-}$  2D2 Ly5.1 CD4<sup>+</sup> T cells (Fig. 3A). The following day, mice were immunized with MOG-CFA to induce EAE. In this adoptive transfer model, 2D2 CD4<sup>+</sup> T cells, but not the bystander CD4<sup>+</sup> T cells, cause EAE (48, 49). Moreover, only the bystander CD4<sup>+</sup> T cells expressed ER $\alpha$  and were therefore the only cells able to respond to E2 treatment (Fig. 3A). Upon immunization, strong and rapid EAE developed in mice transferred with ER $\alpha^{KO}$  2D2 T cells in the absence of E2 treatment, whether the cotransferred bystander CD4<sup>+</sup> T cell population expressed ER $\alpha$  or not (Fig. 3B). By contrast, in the presence of E2, disease was significantly less severe in mice injected with WT ER $\alpha^{fl/fl}$  CD4<sup>+</sup> T cells as compared with mice that received ER $\alpha$ -deficient CD4<sup>+</sup> T cells (Fig. 3C). Disease protection in this group was associated with reduced inflammatory infiltrates in the spinal cord as shown by anti-CD45 staining on spinal cord section by confocal microscopy

(Fig. 3D) and FACS analysis of inflammatory leukocytes extracted from the spinal cords (Fig. 3E). Absolute numbers of CNS-infiltrating CD4<sup>+</sup> T cells, including pathogenic ROR $\gamma$ T<sup>+</sup> 2D2 CD45.1<sup>+</sup> T cells, were strongly reduced in mice protected from EAE because of the cotransfer of ER $\alpha$ -responsive bystander CD4<sup>+</sup> T cells (Fig. 3F).

Similar results were obtained in this adoptive transfer model using low numbers of ER $\alpha^{KO}$  2D2 T cells (Supplemental Fig. 2). As shown in Supplemental Fig. 2, the absolute numbers of 2D2<sup>+</sup> T cells were selectively reduced in WT B6 ER $\alpha^{fl/fl}$  mice but not in mice lacking ER $\alpha$  in T lymphocytes. The MOG<sub>35–55</sub>-specific production of IL-17, GM-CSF, and IFN- $\gamma$  was significantly inhibited in E2-treated WT mice but not in E2-treated CD4<sup>Cre</sup> ER $\alpha^{fl/fl}$  mice (Supplemental Fig. 2). In agreement with the data in Figs. 1, 2, E2 treatment significantly reduced the frequency and absolute numbers of IL-17-producing 2D2 CD4<sup>+</sup> T cells in WT B6 ER $\alpha^{fl/fl}$  mice but not in mice lacking ER $\alpha$  in T lymphocytes (Supplemental Fig. 2). This model was then used throughout the rest of the study.

**FIGURE 6.** Similar EAE course in Rag<sup>KO</sup> mice cotransferred with CD4<sup>+</sup> T cells lacking or not ER $\alpha$  in Foxp3<sup>+</sup> Treg cells in absence of E2 supplementation. **(A)** Immunodeficient ER $\alpha$ <sup>KO</sup>Rag<sup>KO</sup> mice were transferred with 2D2 T cells together with CD4<sup>+</sup> T cells purified from Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup> or control ER $\alpha$ <sup>fl/fl</sup> mice. The day following T cell transfer, mice were immunized with MOG peptide in CFA to induce EAE. Mice were daily monitored for clinical signs of EAE, and the CDI was calculated for each mouse. Error bars show mean  $\pm$  SEM. **(B)** Representative flow cytometry of CD45<sup>+</sup> cells, CD4<sup>+</sup> T cells, and 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells in the spinal cord (SC) 25 d post-disease induction. **(C)** Representative flow cytometry of intracellular production of IFN- $\gamma$ , IL-17, and GM-CSF in 2D2 CD4<sup>+</sup> T cells of SC after PMA/ionomycin stimulation. n.s., not significant.



#### ER $\alpha$ signaling in bystander CD4<sup>+</sup> Foxp3<sup>neg</sup> T cells is necessary and sufficient to inhibit pathogenicity of Th17 cell in trans

As bystander CD4<sup>+</sup> T cells contain Foxp3<sup>+</sup> Treg cells, we sought to determine whether E2/ER $\alpha$  signaling in this subset was important or not to mediate EAE protection. Therefore, we generated mice selectively lacking ER $\alpha$  in Treg cells using a reported/deleter mouse model (45) as described (Fig. 4A). As expected, genomic Cre-mediated deletion of the targeted *Esr1* exon was selectively observed in Treg cells (CD25<sup>+</sup>CD45RB<sup>low</sup>CD4<sup>+</sup>) from Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup> mice but not in naive CD4 T cells (CD45RB<sup>high</sup>), CD8 T cells, or B cells (Fig. 4B). Male Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup> and female Foxp3<sup>YFP-Cre</sup>/YFP-CreER $\alpha$ <sup>fl/fl</sup> mice were born at a Mendelian frequency and lacked splenomegaly, lymphadenopathy, or other clinical signs of autoimmune pathology up to 14 wk of age. Flow cytometric analyses revealed unaltered frequencies and absolute numbers of thymic and peripheral CD8<sup>+</sup> and CD4<sup>+</sup> T cells, including Foxp3<sup>+</sup> CD4<sup>+</sup> T cell subsets in the mesenteric LN or spleen in female Foxp3<sup>YFP-Cre</sup>/YFP-CreER $\alpha$ <sup>fl/fl</sup> mice (Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup>) as compared with Foxp3<sup>YFP-Cre</sup>/YFP-CreER $\alpha$ <sup>+/+</sup> littermate controls (Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>+/+</sup>) (Fig. 4C).

We next examined the effect of E2 treatment on the in vivo priming of 2D2 T cells in recipient Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup> mice. Again, although the relative frequency of CD4<sup>+</sup> T cells or 2D2 T cells in draining lymph nodes was not affected by E2 treatment (Fig. 5A), a dramatic downregulation in the production of IFN- $\gamma$  and IL-17 by MOG-stimulated 2D2 CD4<sup>+</sup> T cells was observed in E2-treated mice (Fig. 5B). Likewise, E2 treatment significantly reduced the frequency and absolute numbers of IL-17-producing 2D2 CD4<sup>+</sup> T cells (Fig. 5C) as well as the development of CCR6<sup>+</sup>ROR $\gamma$ t<sup>+</sup> Th17 cells (Fig. 5D). Thus, ER $\alpha$  signaling in Foxp3<sup>+</sup> Treg cells is dispensable for E2-mediated inhibition of Th17 cell priming in lymph nodes.

Using the adoptive transfer model described in Fig. 3A, we then compared the capacity of bystander CD4<sup>+</sup> T cells in which ER $\alpha$  was deleted in Foxp3<sup>+</sup> Treg cells (Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup>) to inhibit

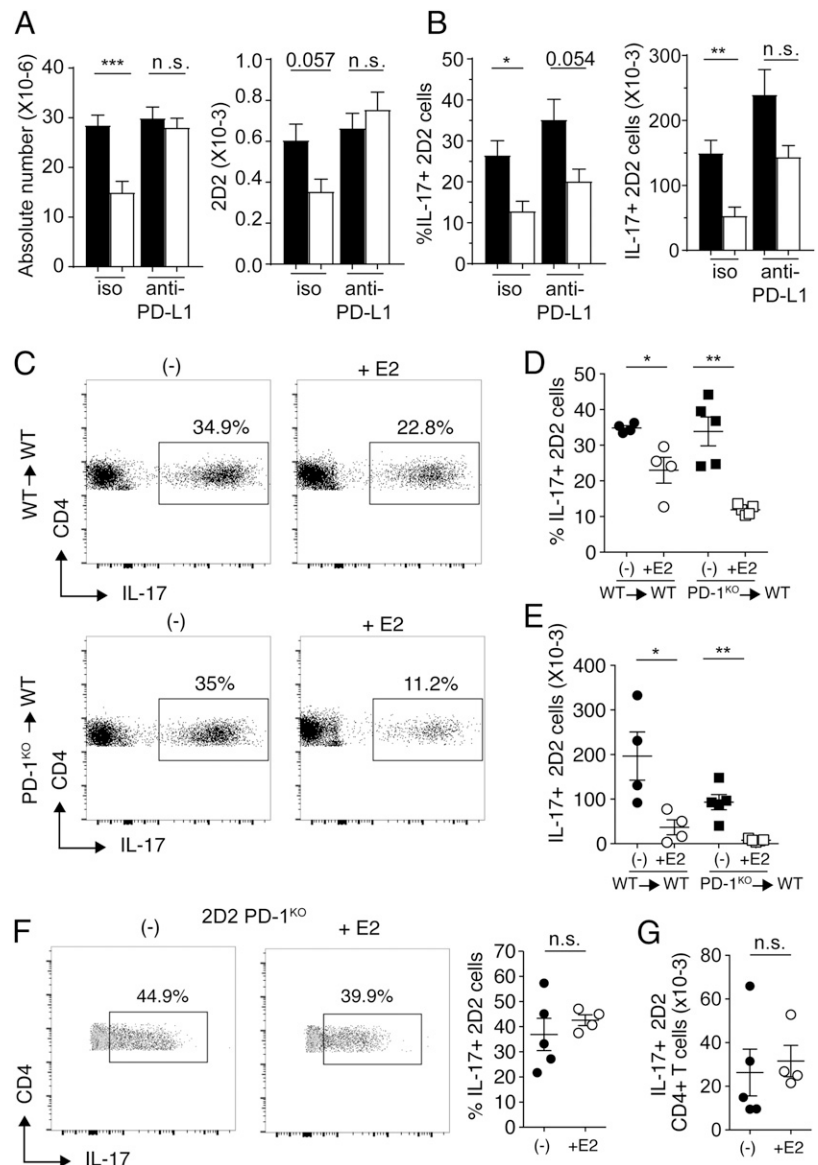
EAE induced by 2D2 Ly5.1<sup>+</sup>ER $\alpha$ <sup>KO</sup>CD4<sup>+</sup> T cells in the presence of E2. In accordance with our previous experiments, strong EAE developed in mice coinjected with ER $\alpha$ -deficient CD4<sup>+</sup> T cells starting at day 9 postimmunization, whereas the other groups that received WT or Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup>CD4 T cells were significantly protected (Fig. 5E). Unexpectedly, the protective effect of bystander CD4<sup>+</sup> T cells was further enhanced when ER $\alpha$  was deleted in Foxp3<sup>+</sup> Treg cells as compared with WT CD4<sup>+</sup> T cells (Fig. 5E). In agreement with clinical EAE, the absolute numbers of CD45<sup>+</sup> inflammatory infiltrates in the spinal cord cells were strongly reduced in mice injected with Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup>CD4<sup>+</sup> T cells as compared with E2-unresponsive CD4<sup>Cre</sup>ER $\alpha$ <sup>fl/fl</sup> T cells, whereas mice injected with WT CD4 T cells showed an intermediate phenotype (Fig. 5F). By contrast, no disease protection was observed in mice cotransferred with CD4<sup>+</sup> T cells from Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>fl/fl</sup> or control Foxp3<sup>YFP-Cre</sup>ER $\alpha$ <sup>+/+</sup> mice in the absence of E2 supplementation (Fig. 6A). In this experimental setting, inflammatory infiltrates were qualitatively similar in both groups as well as the frequencies of CNS-infiltrating 2D2 CD4 T cells producing IFN- $\gamma$ , IL-17, and/or GM-CSF (Fig. 6B, 6C). Thus, in absence of sustained E2 supplementation, bystander CD4 T cells do not suppress cognate 2D2 T cell activation and EAE development whether they express ER $\alpha$  in Foxp3<sup>+</sup> Treg cells or not (Fig. 6). Altogether, our results demonstrate that estrogen signaling through ER $\alpha$  within bystander Foxp3<sup>neg</sup>CD4<sup>+</sup> T cells is necessary and sufficient to restrict differentiation of encephalitogenic Th17 cells in trans and protect from CNS autoimmunity.

#### The PD-1/PD-L1 axis is required for E2-mediated Th17 cell inhibition

Previous works have shown that protective action of E2 on EAE was lost in mice lacking either PD-1 (9) or PD-L1 (39); we therefore investigated whether a functional PD-1/PD-L1 axis was required for the E2-mediated inhibition of Th17 priming. E2 treatment strongly decreased total cell numbers in draining



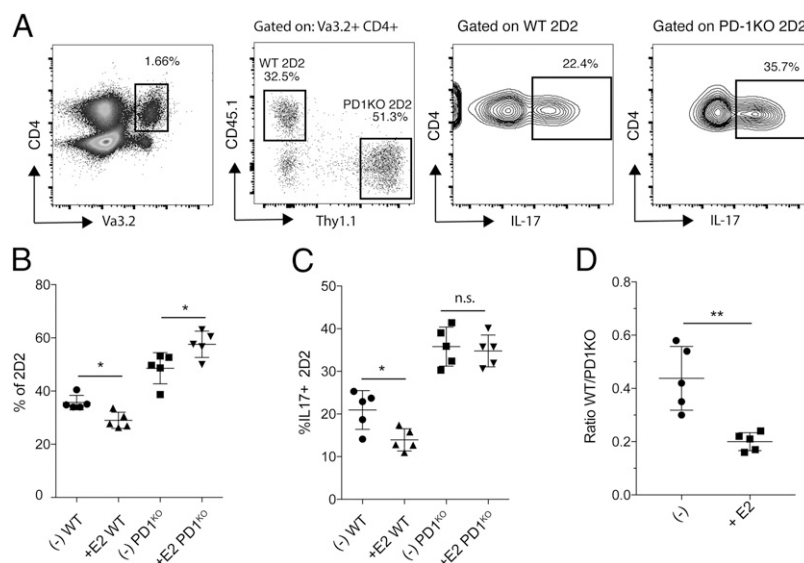
**FIGURE 7.** PD-1 expression on cognate T cells but not on bystander T cells is necessary for the inhibition of Th17 differentiation *in vivo* by E2. (**A** and **B**) B6 mice were implanted (open bar) or not (filled bar) with E2 pellet as in Fig. 1. One day before immunization with MOG<sub>35-55</sub> in CFA, mice were adoptively transferred with naive 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> T cells. Mice received three i.p. injection of either isotype control or anti-PD-L1 Abs (100  $\mu$ g/mouse) 1 d before immunization and 2 and 4 d after immunization. (**A**) At day 6, the absolute number of LNC and 2D2 CD45.1<sup>+</sup> CD4<sup>+</sup> was assessed. Error bars show mean  $\pm$  SEM; this graph represents a pool of two independent experiments,  $n = 9$  or 15 mice per group.  $^{**}p < 0.01$ ,  $^{****}p < 0.0001$ , one-way ANOVA. (**B**) Frequency and absolute number of IL-17<sup>+</sup> 2D2 T cells was assessed in dLN after PMA/ionomycin stimulation by flow cytometry. Error bars show mean  $\pm$  SEM; this graph represents a pool of two independent experiments,  $n = 9$  or 15 mice per group.  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , one-way ANOVA. (**C** and **D**) Bone marrow cells from WT or PD-1<sup>KO</sup> mice were injected into lethally irradiated B6 mice. Eight weeks after reconstitution, mice were implanted or not with E2 pellet. One day before immunization MOG<sub>35-55</sub> in CFA, mice were adoptively transferred with naive CD45.1<sup>+</sup> 2D2 CD4<sup>+</sup> T cells. (**C** and **D**) At day 6, the frequency of IL-17<sup>+</sup> 2D2 CD45.1<sup>+</sup> T cells was assessed after PMA/ionomycin stimulation by intracellular staining. Error bars show mean  $\pm$  SEM,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , Mann-Whitney *U* test. (**E**) Absolute number of IL-17<sup>+</sup> 2D2 T cells. Error bars show mean  $\pm$  SEM,  $^{*}p < 0.05$ ,  $^{**}p < 0.01$ , Mann-Whitney *U* test. (**F** and **G**) B6 mice were implanted or not with 2.5 mg of E2 pellet 1 wk before immunization with MOG<sub>35-55</sub> in CFA. One day before immunization, mice were adoptively transferred with naive CD4<sup>+</sup> T cells from CD45.1<sup>+</sup> 2D2 TCR transgenic mice deficient for PD-1. (**F**) At day 6, the frequency of IL-17<sup>+</sup> 2D2 T cells was assessed by intracellular staining. Error bars show mean  $\pm$  SEM. (**G**) Absolute number of IL-17<sup>+</sup> 2D2 T cells. Error bars show mean  $\pm$  SEM. dLN, draining lymph node; n.s., not significant.



lymph nodes, including 2D2 CD4<sup>+</sup> T cells (Fig. 7A). This anti-inflammatory effect of E2 was lost in recipient mice treated with PD-L1 blocking mAb (Fig. 7A). In these mice, the frequency and absolute numbers of IL-17-producing 2D2 CD4<sup>+</sup> T cells are similar to the values obtained in control untreated mice (Fig. 7B). Although, we still noticed a trend toward an inhibitory action of E2 in anti-PD-L1 treated mice; this was NS (Fig. 7B). As E2-dependent upregulation of PD-1 on CD4<sup>+</sup> T cells has been previously reported (50), we next determined whether expression of PD-1 on bystander CD4<sup>+</sup> T cells was involved in the *trans* inhibition of Th17 cell priming. Chimeric mice reconstituted with PD-1-deficient bone marrow cells were used as recipients. The frequency of Foxp3<sup>+</sup> Treg cells was similar in draining lymph nodes from WT and PD-1-deficient bone marrow chimeras. In both groups, E2 treatment did not upregulate the frequency of Foxp3<sup>+</sup> Treg cells, whether they express or not PD-1 molecules (Supplemental Fig. 3A, 3B). As expected, we noticed a strong upregulation of PD-1 on 2D2 CD4<sup>+</sup> T cells by day 6 postimmunization as a consequence of MOG-driven T cell activation *in situ* (Supplemental Fig. 3C). No differences were observed between control and E2-treated mice, indicating that MOG-driven 2D2 CD4<sup>+</sup> T cell activation was not affected by

estrogen signaling. By contrast, the frequency (Fig. 7C, 7D) and absolute numbers (Fig. 7E) of IL-17-producing 2D2 CD4<sup>+</sup> T cells were significantly inhibited in both E2-treated WT and PD-1<sup>KO</sup> chimeras.

As PD-1/PD-L1 pathway has been suspected to play a role in E2-mediated EAE protection (9), we next investigated whether PD-1 expressed on activated 2D2 CD4<sup>+</sup> T cells was required for the inhibition of the Th17 response. PD-1-deficient naive 2D2 CD4<sup>+</sup> T cells (47) were adoptively transferred in mice treated or not with E2, and the development of IL-17-producing 2D2 T cells was monitored in draining lymph nodes. Contrasting with our results with PD-1-sufficient 2D2 cells, the frequency of IL-17<sup>+</sup> cognate T cells was not affected by E2 treatment when 2D2 cells were lacking PD-1 (Fig. 7F, 7G). To establish further the cell-intrinsic role of PD-1 on 2D2 CD4<sup>+</sup> T cells, we performed adoptive co-transfer experiments of naive CD4<sup>+</sup> T cells from either Ly5.1 WT and Thy1.1 PD-1<sup>KO</sup> 2D2 mice into Ly5.2 B6 mice treated or not with E2 (Fig. 8). Mice were immunized with MOG-peptide in CFA, and the draining lymph nodes were collected at day 6. LNC were stimulated with PMA-ionomycin, and the frequency of IL-17-producing cells among CD45.1<sup>+</sup> WT and Thy1.1<sup>+</sup> PD-1<sup>KO</sup> 2D2 T cells were assessed as shown in Fig. 8A. We observed



**FIGURE 8.** PD-1 expression on cognate 2D2 T cells acts in a cell-intrinsic manner to promote E2-mediated inhibition of Th17 cell differentiation. B6 mice were implanted or not with E2 pellet as in Fig. 1. Naive 2D2 CD4<sup>+</sup> T cells purified from Ly5.1<sup>+</sup> WT and Thy1.1<sup>+</sup> PD-1<sup>KO</sup> mice were cotransferred (ratio 1:1) 1 d before immunization with MOG<sub>35–55</sub> in CFA. Draining LNC were analyzed at day 6 postimmunization. **(A)** Flow cytometry staining representing WT (CD45.1<sup>+</sup>) and PD-1<sup>KO</sup> (Thy1.1<sup>+</sup>) 2D2 CD4<sup>+</sup> T cells among CD4<sup>+</sup> Va3.2<sup>+</sup> cells (on the left) and their relative IL-17<sup>+</sup> frequencies (on the right). **(B)** The frequency of WT and PD1<sup>KO</sup> 2D2 T cells for each individual mouse from control or E2-treated groups is shown. **(C)** The frequency of IL-17–producing cells among WT and PD1<sup>KO</sup> 2D2 T cells was assessed after PMA/ionomycin stimulation by intracellular staining as shown in (A) left panels. **(D)** Results from panel (B) and (C) were integrated to calculate the relative number of IL-17<sup>+</sup> 2D2 T cells expressing the allotypic markers CD45.1 (WT) or Thy1.1 (PD-1<sup>KO</sup>). For each individual mouse, these numbers were then expressed as a ratio between WT and PD-1<sup>KO</sup> T cells. The decreased ratio in E2-treated mice shows that Th17 development was selectively inhibited among the PD-1–sufficient 2D2 T cells. Error bars show mean ± SD, \**p* < 0.05, \*\**p* < 0.01, Mann–Whitney *U* test. n.s., not significant.

a reduction in the relative frequency of WT 2D2 T cells in E2-treated mice relative to PD-1–deficient 2D2 T cells (Fig. 8B). This was associated with a selective inhibition in the frequency of IL-17–producing cells among the PD-1–sufficient 2D2 T cells (Fig. 8C). Indeed, the WT/PD-1<sup>KO</sup> Th17 cell ratio was significantly decreased in mice treated with E2 compared with untreated controls (Fig. 8D). These results are consistent with the conclusion that within the same individual mice, E2 treatment selectively inhibited the Th17 differentiation and expansion of PD-1–sufficient 2D2 T cells, whereas PD-1<sup>KO</sup> cells were largely unaffected.

Together, these data show that cell-intrinsic PD-1 signaling within cognate 2D2 T cells, but neither within endogenous CD4<sup>+</sup> T cells nor Treg cells, is required for the inhibition of Th17 cell priming by E2-sensitized bystander CD4<sup>+</sup> T cells.

## Discussion

We reported previously a requirement for ER $\alpha$  expression in hematopoietic cells, namely T cells rather than DCs or myeloid cells, in the anti-inflammatory action of E2 administration (10). This suppressive effect, characterized by inhibition of Th1/Th17 cell priming in inflammatory lymph nodes, was elicited at steady-state serum concentrations of E2 congruent with pregnancy values. We describe in this paper an indirect path through ER $\alpha$  signaling in noncognate conventional CD4<sup>+</sup> T cells as the main mechanism by which estrogens limit Th1/Th17 cell differentiation of cognate CD4<sup>+</sup> T cells *in trans* and protect from EAE. We show that ER $\alpha$  activation in MOG-specific CD4<sup>+</sup> T cells is totally dispensable to this action. This mechanism appears to selectively limit acquisition of Th17 effector functions in cognate CD4<sup>+</sup> T cells but not their potential for Th2 development nor their proliferative response upon subsequent *ex vivo* challenge. *In vivo* experiments on Rag2<sup>KO</sup>ER $\alpha$ <sup>KO</sup> mice showed that estrogen-sensitized ER $\alpha$ –proficient CD4<sup>+</sup> T cells, unlike their ER $\alpha$ –deficient counterparts, inhibited the development of Th17 cell–mediated EAE when

coadministered with naive ER $\alpha$ –deficient 2D2 T cells. Bystander CD4<sup>+</sup> T cells from mice lacking *Esr1* gene specifically in Treg cells were still highly proficient in their capacity to inhibit Th17 cell differentiation and EAE development. Actually, we consistently observed an enhanced capacity of these cells to suppress EAE as compared with WT CD4<sup>+</sup> T cells. These results might suggest that in the context of inflammation, ER $\alpha$  signaling in Treg cells could negatively impact on Treg cell stability or regulatory potential. Indeed, a recent study has suggested that ER $\alpha$  may suppress Foxp3 expression and iTreg development both *in vivo* and *in vitro* (51). Altogether, our data bring to light an important suppressive property of estrogen by which ER $\alpha$  signaling in bystander Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells promotes their capacity to restrict the differentiation of cognate autoantigen-specific Th17 cells *in trans*, thereby protecting from CNS autoimmunity.

The notion that bystander noncognate Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells can drastically influence the differentiation of the Ag-specific Th17 cells is not without precedent (48, 52). In an elegant study, Hirahara and colleagues (48) demonstrated that IL-27–primed nonspecific bystander T cells could limit Th17 cell differentiation of Ag-specific effector cells *in trans*. They provided evidence that APC-derived IL-27 acted on conventional naive CD4<sup>+</sup> T cells to promote Stat-1–dependent PD-L1 expression. IL-27–primed noncognate PD-L1<sup>+</sup> CD4<sup>+</sup> T cells were able to restrict Th17 cell development and to protect from EAE through PD-1 expressed on cognate T cells (48). More recently, it was reported that type I IFN signaling in T cells could promote the development of FoxA1 Treg cells able to suppress autoreactive T cells in the CNS, again through PD-1/PD-L1 interactions (52). The PD-1/PD-L1 axis has also been suspected to play a role in E2-mediated EAE protection. Indeed, previous works have shown that protective action of E2 on EAE was lost in mice lacking either PD-1 (9) or PD-L1 (39). It was initially suggested that the anti-inflammatory effects of E2 were mediated through its capacity to

drive expansion of Treg cells in vivo (37) and to enhance PD-1 expression on various cell types, including APCs (53) or Foxp3<sup>+</sup> Treg cells (54). Our present results invalidate these assumptions and demonstrate that PD-1 expression on cognate CD4<sup>+</sup> T cells, but not on bystander CD4<sup>+</sup> T cells nor APCs, is required for the inhibition of Th17 differentiation by E2. Indeed, in cotransfer experiments we clearly showed that only PD-1-sufficient 2D2 T cells were amenable to E2-dependent inhibition of Th17 development, whereas PD-1<sup>KO</sup> 2D2 Th17 cells that developed in the same inflammatory lymph nodes were not affected (see Fig. 8). Thus, induction of Treg cells through PD-1 engagement of cognate 2D2 T cells is therefore unlikely to contribute to the inhibitory effect of E2. These results, rather, support the conclusion that the restriction of Th17 cell development by E2-primed bystander CD4<sup>+</sup> T cells requires cell-intrinsic PD-1 signaling within cognate 2D2 T cells. Indeed, PD-1/PD-L1 interactions have been shown to impair Th17 differentiation in mouse models (48) and in patients with *STAT3* loss- and *STAT1* gain-of-function mutations (55). Whether PD-1 on MOG-specific 2D2 T cells interacts with PD-L1 expressing bystander CD4<sup>+</sup> T cells (48, 52) or others, PD-L1 expressing cells in draining lymph nodes to inhibit encephalitogenic Th17 cell development will deserve further investigations.

In conclusion, our data now provide direct evidence that bystander Foxp3<sup>neg</sup> CD4<sup>+</sup> T lymphocytes are the main ER $\alpha$ -expressing target cells orchestrating the anti-inflammatory effects of E2 in EAE. This involves the inhibition in *trans* of cognate Th17 cell development in inflammatory lymph nodes through a mechanism that is dependent on PD-1 expression on cognate but not bystander CD4<sup>+</sup> T cells. Harnessing ER $\alpha$  signaling in Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells may therefore represent a valuable therapeutic approach to limit inflammation in multiple sclerosis. ER modulation strategy could be possibly combined with regulatory cytokines, such as IFN- $\beta$ , to promote synergistic or additive anti-inflammatory effects on multiple sclerosis, as it has been reported in a recent clinical trial (14). Delineating further the molecular mechanisms by which ER $\alpha$  signals in Foxp3<sup>neg</sup> CD4<sup>+</sup> T cells may help to optimize ER modulation strategies, to limit the deleterious impact of estrogen on reproductive tissues, and to improve the protective action of estrogens in CNS autoimmunity in an effort to mimic the beneficial effect of pregnancy in women with multiple sclerosis.

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## Disclosures

The authors have no financial conflicts of interest.

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