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CUTTING EDGE

Cutting Edge: Estrogen Drives Expansion of the $CD4^+CD25^+$ Regulatory T Cell Compartment[1]

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$CD4^+CD25^+$ regulatory T cells are crucial to the maintenance of tolerance in normal individuals. However, the factors regulating this cell population and its function are largely unknown. Estrogen has been shown to protect against the development of autoimmune disease, yet the mechanism is not known. We demonstrate that estrogen (17-β-estradiol, E2) is capable of augmenting FoxP3 expression in vitro and in vivo. Treatment of naive mice with E2 increased both $CD25^+$ cell number and FoxP3 expression level. Further, the ability of E2 to protect against autoimmune disease (experimental autoimmune encephalomyelitis) correlated with its ability to up-regulate FoxP3, as both were reduced in estrogen receptor α-deficient animals. Finally, E2 treatment and pregnancy induced FoxP3 protein expression to a similar degree, suggesting that high estrogen levels during pregnancy may help to maintain fetal tolerance. In summary, our data suggest E2 promotes tolerance by expanding the regulatory T cell compartment. The Journal of Immunology,* 2004, 173: 2227–2230.

Although induction of autoimmune disease involves many factors, the defining event is the loss of T cell tolerance to self-Ags. Tolerance is maintained in part by negative selection of autoreactive T cells in the thymus and by induction of anergy in the periphery (1, 2). However, these two mechanisms alone are insufficient to preserve the tolerant state. Recently, the crucial role of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg})[4] in suppression of responses to self-Ags has been demonstrated in both mice and humans (3–5). Failures in the function of the T_{reg} compartment can therefore be responsible for the development of autoimmune disease, and enhancing its function may represent a therapeutic strategy.

The observation that autoimmune diseases occur more frequently in females than in males led to investigation of the role of sex hormones in maintenance of immune tolerance (6). Previous work in our lab has shown that estrogen (E2) treatment protects mice from development of experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis (7). However, the mechanism of this effect has not yet been fully characterized.

FoxP3 is a transcriptional repressor required for the development and function of T_{reg} (8). Deficiency of FoxP3 leads to autoimmune diseases including X-linked immune dysfunction, polyendocrinopathy, and enteropathy in humans and scurfy in mice. Although CD4 and CD25 partially identify the T_{reg} compartment, FoxP3 is currently the most definitive marker of regulatory function (8, 9, 10). Therefore, we investigated whether E2 treatment might exert its EAE-protective effect by increasing FoxP3 expression in $CD4^+$ T cells. In this report, we show that E2 treatment alone is sufficient to expand the T_{reg} compartment in vivo. In addition, we present evidence that E2 induces FoxP3 in $CD4^+CD25^-$ T cells in vitro.

Materials and Methods

Mice

Female naive or syngeneic pregnant (19 days) C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Estrogen receptor α knockout (*Esr1*$^{-/-}$) mice were purchased from Taconic Farms (Germantown, NY). Most experiments represent cells pooled from at least five mice per experimental condition. Animals were housed and cared for according to institutional guidelines in the Animal Resource Facility at the Veterans Affairs Medical Center (Portland, OR).

Hormone treatment

For E2 therapy, a 3-mm pellet containing 2.5 or 15 mg (as indicated) 17-β-estradiol (Innovative Research of America, Sarasota, FL) was implanted s.c. (dorsally) 7 days before immunization (EAE) or 14 days before analysis of naive mice. These pellets are designed to release their contents at a constant rate over 60 days. Control animals were implanted with pellets containing saline. Serum levels of E2 were monitored by RIA as described (7).

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[4] Abbreviations used in this paper: T_{reg}, regulatory T cell; E2, 17-β-estradiol; EAE, experimental autoimmune encephalomyelitis; RE, relative expression; MOG, myelin oligodendrocyte glycoprotein; TFIIB, transcription factor IIB.

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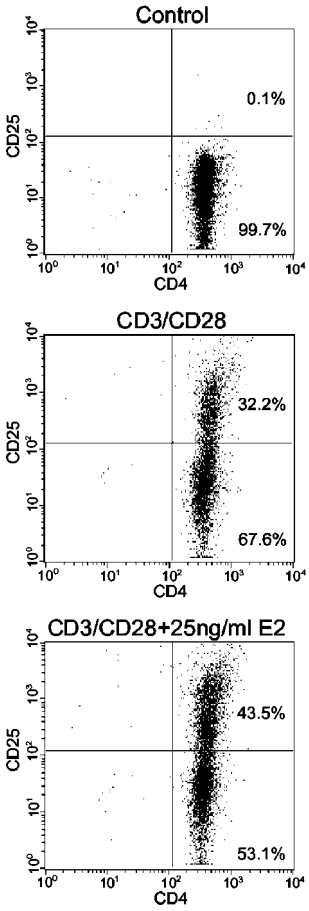

FIGURE 1. Flow cytometric analysis comparing $CD4^+CD25^-$ T cells treated as indicated in vitro for 24 h. Control sample is $CD4^+CD25^-$ cells cultured for 24 h without treatment. Plots show CD25-PE on the *y*-axis and CD4-FITC on the *x*-axis.

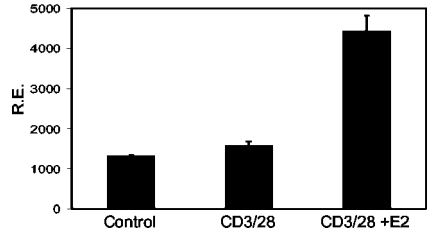

FIGURE 2. Real-time RT-PCR analysis of *Foxp3* expression in cells from Fig. 1. Foxp3 levels are shown relative to housekeeping gene L32. Error bars are SD of triplicate samples. One representative result of three is shown.

Induction of EAE

Immunization and clinical evaluation were performed as described previously (7, 11). Briefly, mice were immunized s.c. in the flanks with 200 μg of myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide in CFA (Difco Laboratories, Detroit, MI). Mice were also given pertussis toxin i.p on days 0 (75 ng) and 2 (200 ng).

Cell preparation and culture

Single cell suspensions were prepared from spleens and RBCs lysed. Nonimmunized mice receiving E2 pellets were sacrificed 14 days after implantation, while immunized mice were sacrificed at the peak of EAE disease severity, ~17 days after induction. Purified $CD4^+$ cells were obtained by MACS according to manufacturer's protocols (Miltenyi Biotec, Bergisch Gladbach, Germany). For flow cytometry, cells were stained with FITC-anti-CD4 and PE-anti-CD25 (BD Pharmingen, San Diego, CA). $CD4^+CD25^-$ cells were obtained from purified $CD4^+$ using a FACSVantage (BD Immunocytometry Systems, San Jose, CA). For in vitro experiments, $CD4^+CD25^-$ cells were stimulated with 5 μg/ml anti-CD3ϵ and 1 μg/ml anti-CD28 (145-2C11 and 37.51, respectively; BD Pharmingen). In some experiments, cells were treated with E2 alone in the absence of Ab stimulation.

Evaluation of Foxp3 expression

For real-time RT-PCR analysis, total RNA was prepared using the Total RNeasy kit (Qiagen, Germantown, MD) and cDNA was prepared using random hexamer primers (Invitrogen Life Technologies, Grand Island, NY). *Foxp3* message expression was quantified using the ABI 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was performed in a total volume of 25 μl for 40 cycles and products were detected using SYBR Green I dye (Molecular Probes, Eugene, OR). Samples were run in triplicate and relative expression level was determined by normalization to L32 with results presented as relative expression (RE) units. Primer sequences used were as follows: L32, forward: GGA AAC CCA GAG GCA TTG AC; reverse: TCA GGA TCT GGC CCT TGA AC. *Foxp3*, forward: GGC CCT TCT CCA GGA CAG A; reverse: GCT GAT CAT GGC TGG GTT GT. For analysis of FoxP3 protein, cells were washed in PBS then lysed and sonicated in lysis buffer (25 mM Tris, pH 8.5, 2% lithium dodecyl sulfate, 1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium orthovanadate, and 1× complete protease inhibitors (Roche Diagnostic Systems, Mannheim, Germany)) and quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). Lysates were separated on 4–12% gradient bis-Tris gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred to nitrocellulose (GE Osmonics, Minnetonka, MN) followed by blocking in TBS/0.1% Tween 20 with 5% nonfat dry milk. FoxP3 was detected with rabbit-anti-FoxP3 antiserum (12) and standard chemiluminescence. For loading control, blots were stripped and reprobed for transcription factor IIB (TFIIB) (Santa Cruz Biotechnology, Santa Cruz, CA). Positive control lysate was from 293T cells transfected with FoxP3 cDNA. Films were analyzed by volumetric pixel integration using ImageQuant v5.2 (Amersham Biosciences, Uppsala, Sweden).

Results

E2 augments Foxp3 expression in vitro

To determine whether the mechanism by which E2 protects mice from EAE might involve induction or enhancement of T_{reg}, we tested its capacity to induce *Foxp3* expression in vitro in purified (>99%, Fig. 1) $CD4^+CD25^-$ T cells. E2 in combination with TCR stimulation for 24 h induced *Foxp3* mRNA ~2-fold over untreated cell levels, while TCR stimulation alone failed to induce *Foxp3* (Fig. 2). In addition, E2 increased the fraction of $CD25^+$ cells over TCR stimulation alone, consistent with induction of T_{reg} (Fig. 1).

E2 treatment before EAE induction augments Foxp3 expression in vivo

We previously demonstrated that low-dose 17-β-estradiol (E2) treatment dramatically reduces the severity of EAE in mice (7), and that this effect requires estrogen receptor-α (Esr1) (11). To determine whether the ability of E2 to protect mice from EAE correlates with an effect on the T_{reg} compartment, we analyzed *Foxp3* expression levels in the presence or absence of E2 treatment at the peak of disease. Therapeutic doses of E2 significantly increased *Foxp3* mRNA levels in $CD4^+$ T cells from MOG 35–55 peptide-immunized wild-type C57BL/6 mice that were protected from EAE, but not in $CD4^+$ T cells from $Esr1^{-/-}$ mice lacking estrogen receptor-α that developed severe signs of EAE (Fig. 3). In addition, FoxP3 protein expression and $CD25^+$ number in E2-treated mice were substantially lower in $Esr1^{-/-}$ than in wild-type animals (Fig. 4), suggesting a deficient expansion of T_{reg} in the absence of normal E2 responsiveness. Changes in FoxP3 protein level correlated well with changes in $CD25^+$ number in response to E2 by each genotype (Fig. 4).

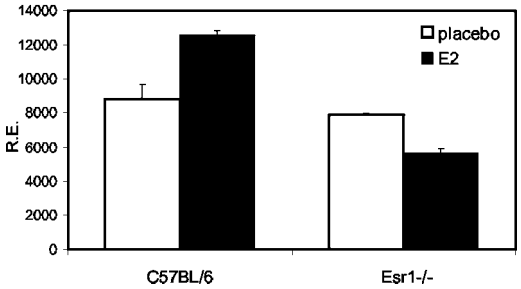

FIGURE 3. Real-time RT-PCR analysis of *Foxp3*. C57BL/6 and Esr1$^{-/-}$ mice were implanted with placebo or 2.5 mg of E2 pellets and immunized 1 wk later with 200 μg of MOG 35–55 peptide in CFA with pertussis toxin on days 0 and +2. At the peak of clinical disease, mice were sacrificed and splenocytes sorted for CD4$^+$ cells. cDNA was prepared and analyzed by real-time PCR to determine Foxp3 mRNA levels. Data are presented as *Foxp3* relative to housekeeping gene L32. Error bars are SD of triplicate samples.

E2 expands the T_{reg} compartment in vivo

Many of the surface markers for T_{reg} (such as CD25 and glucocorticoid-induced TNFR) are also markers of activated effector CD4$^+$ T cells. To avoid a significant contribution of activated T cells to our analysis of the T_{reg} compartment (as in MOG-immunized animals), we treated naive C57BL/6 mice with E2 for 14 days and assessed CD25 and *Foxp3* expression among CD4$^+$ T cells. We observed a significant increase (43%) in the fraction of CD25$^+$ cells among all CD4$^+$ cells (Fig. 5) in E2-treated vs untreated mice. This increase in CD25$^+$ cells was attended by an increase in *Foxp3* mRNA (Fig. 6) and protein (Fig. 7), suggesting that the cells generated are T_{reg} and not activated effector CD4$^+$ cells.

Pregnancy represents a natural instance of sustained high levels of estrogen, as well as a challenge to peripheral tolerance because the fetus bears paternal and alloantigens that can be presented to maternal T cells. It has been reported that pregnancy in humans is attended by an increase in CD4$^+$CD25$^+$ numbers, potentially T_{reg}, yet the signal for this increase is unknown (13, 14). We examined CD4$^+$ T cells from pregnant (19 days) C57BL/6 mice for expression of CD25, *Foxp3* mRNA, and FoxP3 protein. There were significant increases in both the fraction of CD25$^+$ cells (28%, Fig. 5) and the level of FoxP3 protein (Fig. 7). However, there was no significant difference in Foxp3 mRNA level between naive and pregnant mouse CD4$^+$ T cells (Fig. 6).

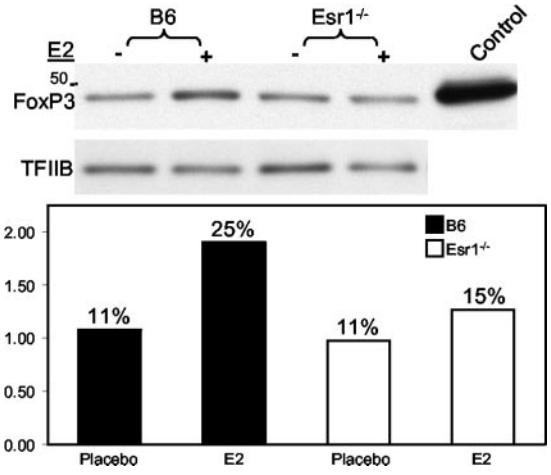

FIGURE 4. FoxP3 Western blot analysis of samples from Fig. 3. Densitometry shows FoxP3 expression level relative to the loading control TFIIB. Control lane is lysate of 293T cells transfected with *Foxp3* cDNA. The CD25$^+$ fraction (among CD4$^+$) of each sample is noted above the bars.

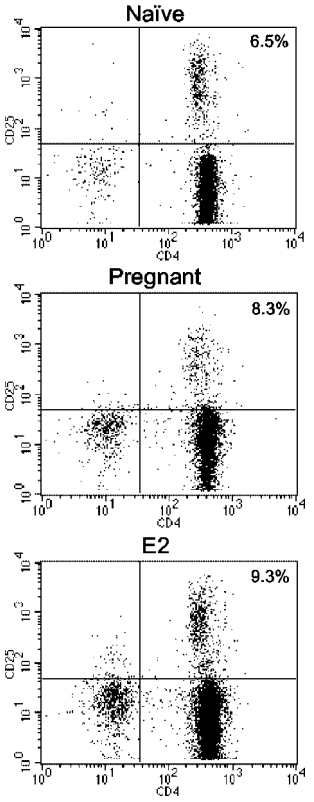

FIGURE 5. Flow cytometric analysis comparing CD4$^+$CD25$^+$ populations in naive (nulliparous), 19 day pregnant, and 14 day E2-treated mice. Quadrant statistics noted are percent of live gate.

Discussion

Identification of signals that regulate the homeostasis, proliferation, and function of T_{reg} is of great importance to understanding the mechanisms by which tolerance breaks down and autoimmunity develops. In the present work, we have identified E2 as a protolerance regulator of the T_{reg} compartment. The ability of E2 to protect mice from EAE correlated with its ability to augment *Foxp3* mRNA and protein expression in the CD4$^+$ T cell compartment. Although these data do not prove that E2 inhibits autoimmunity by expansion of T_{reg}, they do

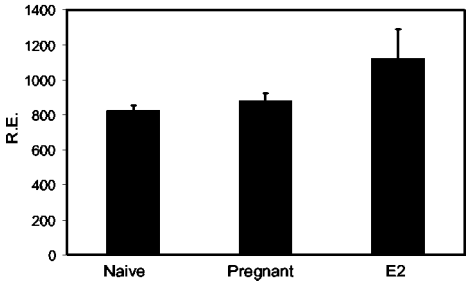

FIGURE 6. Real-time PCR analysis of *Foxp3* mRNA in CD4$^+$ cells from Fig. 5. Data are presented as *Foxp3* relative to housekeeping gene L32. Error bars are SD of triplicate samples.

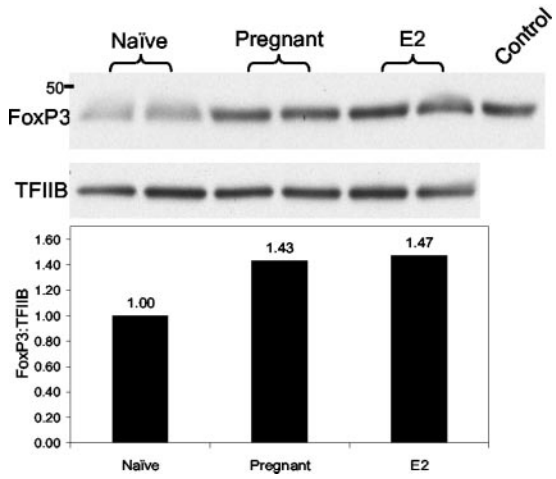

FIGURE 7. FoxP3 Western blot analysis of samples from Fig. 6. Each condition is shown as technical replicate lanes of cells pooled from five mice per condition. Densitometry shows fold induction of FoxP3 relative to naive (nulliparous) untreated mice. Control lane is lysate of 293T cells transfected with *Foxp3* cDNA.

show that E2 is capable of increasing the number of T_{reg}. Because depletion of T_{reg} causes autoimmunity (15) and immune rejection of the fetus (13), however, this is a plausible mechanism.

Importantly, T_{reg} expansion did not require an active autoimmune response, demonstrating that E2 treatment alone in a normal animal is sufficient for this effect. Although the protective outcome may be the same, our results do not distinguish between expansion of the existing T_{reg} population and de novo generation of T_{reg} from non-T_{reg}. To address this question, we tested the latter. Walker et al. (16) have shown previously that $CD4^+CD25^+FoxP3^+$ T_{reg} can be generated from human $CD4^+CD25^-FoxP3^-$ T cells in vitro by activation and prolonged culture, though E2 has never been implicated in this process. We found that E2 in combination with TCR stimulation is capable of increasing *Foxp3* mRNA expression in $CD4^+CD25^-$ T cells. Although TCR stimulation alone provoked a large increase in the number of $CD25^+$ cells, these cells did not express significant *Foxp3*, indicating that they were activated effector cells. In contrast, when $CD4^+CD25^-$ cells were activated in the presence of E2, both the number of $CD25^+$ cells and their *Foxp3* expression level were increased. Thus, E2 may be a signal that redirects peripheral $CD4^+$ T cells toward a $FoxP3^+$ T_{reg} phenotype when activated in a tolerogenic context.

Our demonstration that E2 treatment induces a similar increase in T_{reg} as does pregnancy may provide insight into the natural origin of this effect. A pregnant female is continuously exposed to both self and non-self Ags of fetal origin. The delicate yet powerful tolerance that operates in this context may be responsible not only for preventing rejection of the fetus, but also for protecting the mother from priming against self-Ags in a foreign context and subsequently developing autoimmune disease. Expansion of T_{reg} number and function in response to the high serum estrogen concentration that is concurrent with pregnancy may represent a mode of specific immunosuppression that is invoked only when needed, then switched off postpartum. Of importance, clinical signs in both EAE and multiple sclerosis improve during pregnancy, but tend to recur postpartum.

The autoimmune-protective effects of E2 have been demonstrated in a variety of contexts, yet the mechanism of this effect remains elusive. Work in our laboratory and others suggests that E2 reduces pathological Th1 responses, and this may involve effects on Ag presentation and cytokine production by dendritic cells. However, no effect of E2 has yet been identified that could operate to preserve tolerance before an autoimmune response has been initiated. Our finding that protective E2 treatment in both immunized and naive animals results in expansion of the $CD4^+CD25^+FoxP3^+$ T_{reg} compartment is the first indication that such a mechanism operates in response to a single signal. In addition, this is the first demonstration that the T_{reg} compartment can be expanded in vivo by treatment with a single benign compound. Prior induction or transfer of T_{reg} cells appears to be required to inhibit subsequent activity of encephalitogenic T cells (17, 18). This feature of T_{reg} activity may explain the potent ability of E2 to prevent induction, but not reverse established clinical signs of EAE. Thus, E2 may represent a valuable therapeutic agent for prevention of relapses or progression in a variety of autoimmune diseases because T_{reg} cells have the potential to suppress immune responses to a broad range of self Ags, and hormone therapy is already well-established and tolerated.

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