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Cutting Edge: Estrogen Drives Expansion of the CD4⁺CD25⁺ Regulatory T Cell Compartment

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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 levels. 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) 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 dysregulation, 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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¶ 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 III B.
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 Treg, we analyzed by volumetric pixel integration using ImageQuant v5.2 (Amersham Biosciences, Uppsala, Sweden).

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 Treg 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 Treg 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).
E2 expands the Treg compartment in vivo

Many of the surface markers for Treg (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 Treg 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 Treg 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 Treg, 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).

Discussion

Identification of signals that regulate the homeostasis, proliferation, and function of Treg 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 Treg 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 Treg, they do...
show that E2 is capable of increasing the number of Treg. Because depletion of Treg causes autoimmunity (15) and immune rejection of the fetus (13), however, this is a plausible mechanism.

Importantly, Treg 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 Treg population and de novo generation of Treg from non-Treg. To address this question, we tested the latter. Walker et al. (16) have shown previously that CD4+CD25+Foxp3+ Treg 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+ Treg phenotype when activated in a tolerogenic context.

Our demonstration that E2 treatment induces a similar increase in Treg 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 Treg 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 naïve animals results in expansion of the CD4+CD25+Foxp3+ Treg 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 Treg compartment can be expanded in vivo by treatment with a single benign compound. Prior induction or transfer of Treg cells appears to be required to inhibit subsequent activity of encephalitogenic T cells (17, 18). This feature of Treg 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 Treg 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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