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Progesterone suppresses the mTOR pathway and promotes generation of induced regulatory T cells with increased stability

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While induced FoxP3$^+$ T cells (iTreg cells) are promising cellular therapeutics to treat inflammatory diseases, a limitation in utilizing iTreg cells prepared in vitro is their low stability in inflammatory conditions. Progesterone (P4) is an immune regulatory nuclear hormone with a potent Treg induction activity. We reasoned that this function of progesterone would be utilized to generate iTreg cells with highly suppressive activity and improved stability in vivo. Here we generated iTreg cells with progesterone in vitro and found that progesterone generates iTreg cells that are highly stable in inflammatory conditions. Moreover, P4-induced iTreg cells highly express latency-associated peptide TGF-β1 and are efficient in regulating inflammation in multiple tissues, whereas control iTreg cells induced with TGF-β1 alone are less stable and ineffective in suppressing inflammation. The function of progesterone in inducing iTreg cells with improved regulatory activity is associated with the function of P4 in suppressing the mTOR pathway. Moreover, the function of progesterone in inducing FoxP3$^+$ T cells is decreased but not completely abolished on nuclear progesterone receptor-deficient T cells, suggesting that both nuclear and nonnuclear progesterone receptors are involved in mediating the function. We conclude that P4 can be utilized to generate iTreg cells with a high therapeutic potential in treatment of tissue inflammation.

Keywords: FoxP3 · Inflammation · mTOR · Progesterone · Treg cells

Supporting Information available online

Introduction

FoxP3$^+$ T cells (commonly called Treg cells) are a specialized subset of CD4$^+$ T cells with immune suppressive functions [1, 2]. Treg cells can be made in the thymus from T-cell progenitors as natural Treg cells (nTreg cells) and from naïve T cells as induced Treg cells (iTreg cells) in the periphery. iTreg cells are induced in the periphery in response to various signals including TCR activation, cytokines (IL-2 and TGF-β1), nuclear hormone receptor ligands, and other tissue factors [3–6]. Treg cells produce TGF-β1, IL-10, and/or IL-35, and play essential roles in maintenance of immune tolerance to prevent autoimmune or inflammatory diseases [7–9]. Naïve T cells can become effector T cells that produce IFN-γ, IL-4, and/or IL-17. Among the effector T cells, IL-17-producing T cells (commonly called Th17 cells), while related to Treg

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cells in development in that they are induced by TGF-β1, mount immune responses mainly for immunity and tissue inflammation [10–12].

Because of their suppressive activity on many types of immune cells, Treg cells are considered potential therapeutics to treat inflammatory diseases. Compared to nTreg cells, iTreg cells that are generated in vitro are less stable and readily become effector T cells [13, 14]. In this regard, there is a difference between nTreg cells and iTreg cells in methylation of certain CpG motifs in the Foxp3 locus [15, 16]. nTreg cells, while more stable and effective in suppression, are difficult to prepare in sufficient numbers for therapeutic applications. Some reported that iTreg cells induced in vitro fail to control tissue inflammation [17, 18], whereas others reported significant suppression [19, 20]. It is highly desirable to generate iTreg cells with improved stability in vitro utilizing iTreg-inducing agents. A good example of iTreg-inducing agent is rapamycin [21]. P4 is a nuclear hormone receptor ligand and is a major female sex hormone [22, 23]. It has been reported that P4 has the Treg-inducing function in pregnant mice and can generate iTreg cells from human cord blood naive T cells [24, 25]. We investigated if P4 would be used as a Treg-inducing agent. Our study revealed that P4 can be used to generate iTreg cells with improved stability and function in regulation of tissue inflammation.

Results

P4 induces LAP-TGF-β1-expressing FoxP3$^+$ T cells

While the tolerogenic effect of P4 in expanding murine Treg cells in pregnancy has been reported [25], the induction from naïve T cells, phenotype, and in vivo function of mouse iTreg cells induced by P4 have not been determined. First, we examined the function of P4 in control of naïve mouse T-cell differentiation into iTreg cells. P4 increased the differentiation of naïve T cells into FoxP3$^+$ T cells (Fig. 1A). This process was greatly enhanced in the presence of TGF-β1. We compared multiple T-cell activators such as concanavalin A (Fig. 1A), anti-CD3/CD28 (Fig. 1B), and $OVA_{323\text{-}339}$ peptide (Fig. 1B) for activation of naïve CD4$^+$ T cells in the presence or absence of P4. P4 had a clear Treg-promoting activity with all of the activators. Moreover, the P4-mediated induction occurred when the T cells were activated with a different antigen, myelin oligodendrocyte glycoprotein (MOG) antigen peptide ($MOG_{35\text{-}55}$). Naïve CD4$^+$ T cells from both male and female mice were equally responsive in becoming FoxP3$^+$ T cells in response to P4 (Supporting Information Fig. 1). P4 increased the frequency and absolute numbers of FoxP3$^+$ T cells in a dose-dependent manner in the presence of exogenous TGF-β1 (Fig. 1C). The iTreg cells induced in the presence of P4 (P4-iTreg cells) highly expressed latency-associated peptide (LAP)-TGF-β1 (Fig. 1D), which is the inactive precursor form of active TGF-β1.

P4-iTreg cells were highly efficient in suppressing the proliferation of target T cells (Fig. 2A). P4-iTreg cells were more suppressive than control iTreg cells induced in the absence of exogenous P4. Even P4-iTreg cells induced in the absence of exogenous TGF-β1 were suppressive, suggesting that the suppressive function in vitro is not necessarily dependent on FoxP3 expression. The Treg-specific demethylated region (TSDR) of the FoxP3 gene is unmethylated in nTeg cells but not in iTreg cells [15]. We examined if P4 has any effect on the methylation status of TSDR. The TSDR of P4-iTreg cells was largely methylated and similar to control iTreg cells (Fig. 2B). Thus, P4 does not affect the methylation status of TSDR.

P4 signals through a number of receptors including nuclear progesterone receptors (PR-A and PR-B), membrane progestin receptors (mPRα, mPRβ, and mPRγ), progesterone receptor membrane component-1 and 2 (PGRMC1 and PGRMC2), and glucocorticoid receptor (GR) [26–31]. We examined if PR-A and PR-B are involved in mediating the P4 effect on T-cell differentiation. Significant decreases in induction of FoxP3$^+$ T cells were observed for PR A/B (−/−) T cells compared to wild-type (WT) T cells (Fig. 3A), suggesting a positive role of these receptors. However, P4 had a small but detectable effect on PR A/B (−/−) T cells in generation of iTreg cells, suggesting the potential roles of other receptors. We further examined the frequencies of FoxP3$^+$ T cells in PR A/B (−/−) mice. We found that the frequency of FoxP3$^+$ T cells was reduced specifically in the uterus (Fig. 3B), indicating that the PR effect was largely limited to the P4-regulated uterus, but not other organs, in mice.

P4 suppresses the generation of murine Th17 cells

Development of naïve T cells into Th17 cells versus FoxP3$^+$ T cells is reciprocally regulated by cytokines and other factors. Unlike the shared requirement of TGF-β1, many factors that promote Treg cells would suppress Th17 cells and vice versa. Retinoic acid, vitamin D, and rapamycin are such examples [5, 32, 33]. Because of this, we assessed the role of P4 in induction of IL-17$^+$ CD4$^+$ T cells. We found that P4 dampens mouse T-cell differentiation into Th17 cells driven by TGF-β1 and IL-6 (Fig. 4A). This occurred also with anti-IL-2 that neutralizes IL-2, a cytokine that is known to suppress the Th17 cell development [34]. Next, we examined if PR is involved in the suppression. P4 was able to completely suppress the induction of Th17 cells from PR (−/−) naïve T cells in vitro (Fig. 4B), suggesting that PR is not required for the suppression.

P4 negatively regulates the mTOR signaling pathway

It has been determined that the mammalian target of rapamycin (mTOR) pathway is important for T-cell differentiation. Suppression of mTORC1 (mTOR Complex 1) by rapamycin is implicated in generation of Treg cells [35–38]. We determined if P4 would alter the activity of the mTORC1 pathway. We found that P4 decreased the phosphorylation of S6 ribosomal protein (Fig. 5A), a major substrate of p70 S6 kinase that is a downstream target of the mTORC1 kinase. The function of suppressing the mTOR pathway is consistent with the Treg-inducing activity of P4. These data suggest a possibility that P4 can induce Treg cells through

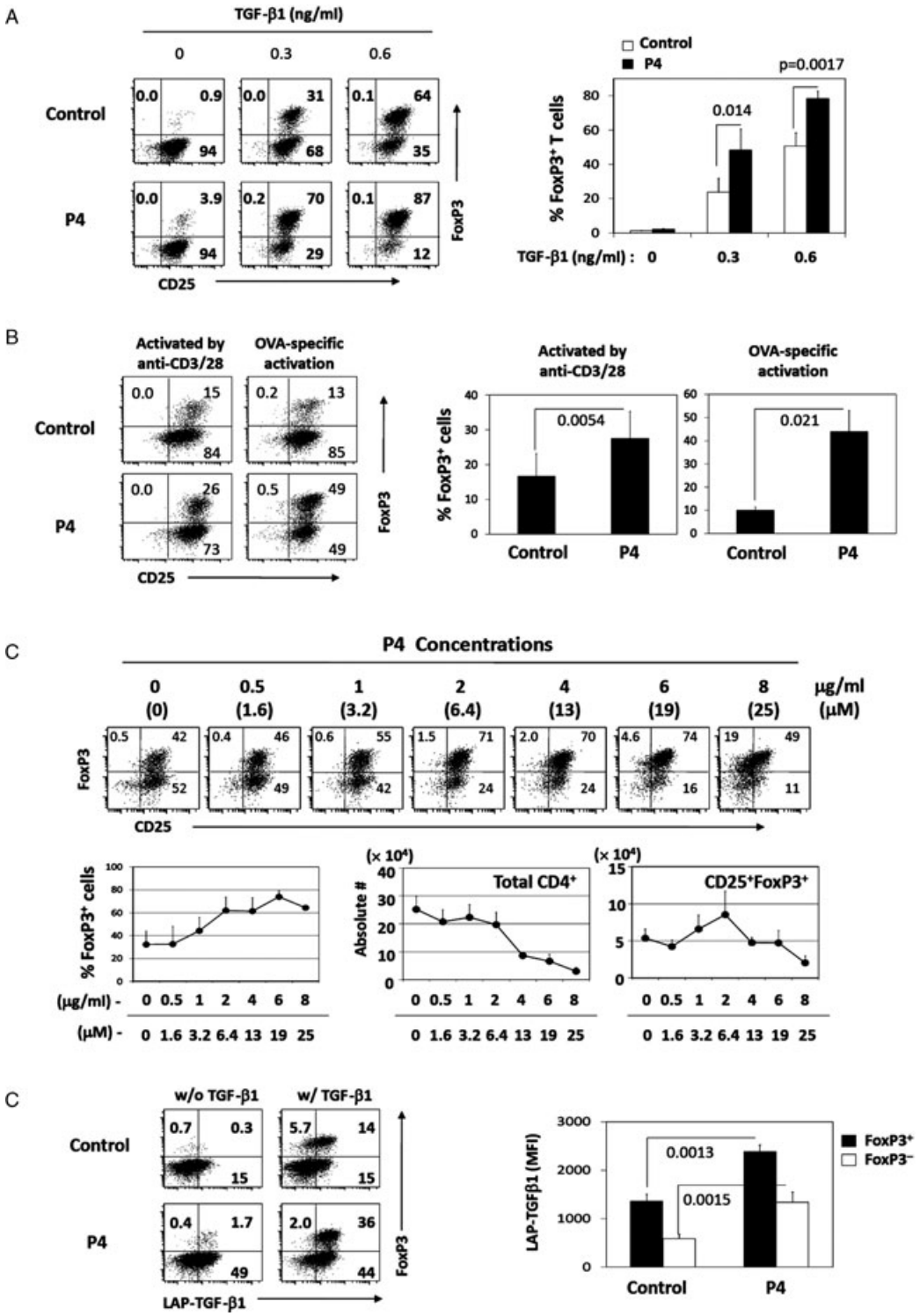

Figure 1. Enhanced induction of FoxP3$^+$ iTreg cells in the presence of P4. (A) Treg-cell-depleted naïve CD4$^+$ T cells were cultured for 6–7 days in the presence or absence of P4 (2 μg/mL) and TGF-β1. Concanavalin A (2.5 μg/mL) and IL-2 (100 U/mL) were added and cells were stained for CD25 and FoxP3 expression. (B) Induction of P4-iTreg cells by activation with anti-CD3/28 antibodies or antigen peptide ($OVA_{323-339}$). Wild-type C57BL/6 Treg-cell-depleted naïve CD4$^+$ T cells were activated with anti-CD3/28 antibodies, and DO11.10 Rag2 (−/−) naïve CD4$^+$T cells were activated with the OVA peptide and irradiated splenocytes. Cells were stained for CD25 and FoxP3 expression (C) Dose-dependent induction of FoxP3$^+$ T cells in response to P4. The cells were cultured in the presence of TGF-β1 and IL-2 and activated with anti-CD3/28 antibodies for 6 days. (D) Induction of LAP-TGF-β1 by P4 on T cells. (B, D) TGF-β1 was used at 0.3 ng/mL. Quantitative data are shown as mean + SEM of 3–8 samples per group pooled from at least three independent experiments; flow cytometry plots are representative. Significant differences ($p < 0.05$) between two groups determined by Student's t-test are shown.

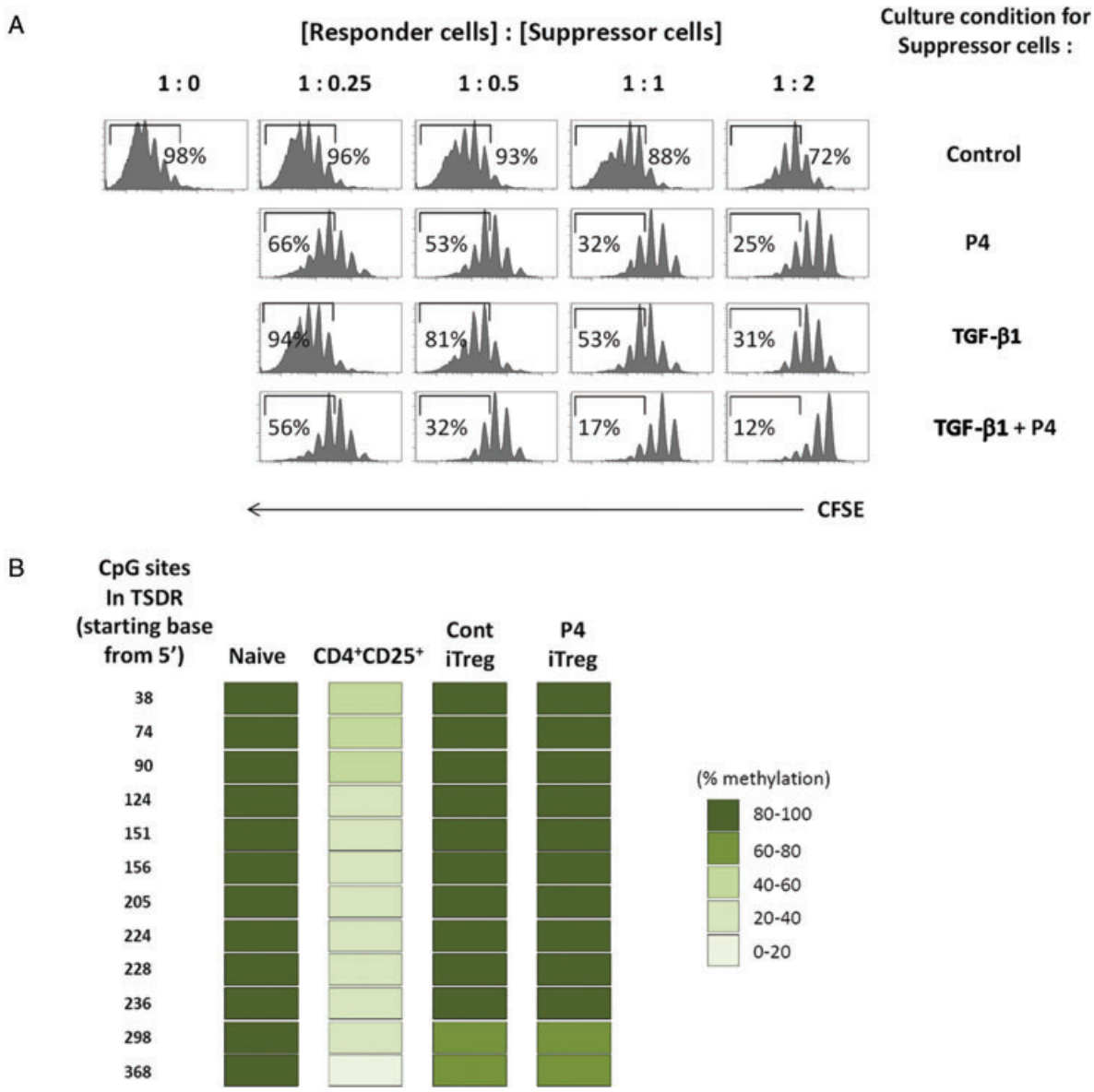

Figure 2. P4 induces iTreg cells with potent suppressive activity but does not induce demethylation in the Treg-specific demethylated region (TSDR) in the *FoxP3* gene. (A) P4-induced iTreg cells were highly suppressive in vitro. Suppression of target T-cell ($CD4^+CD25^-$ responder) proliferation stimulated with anti-CD3 antibodies and irradiated splenic APCs for 3 days by Treg cells was assessed based on dilution of CFSE fluorescence. (B) Methylation of the CpG sites in the TSDR region of nTreg, control Treg, and P4-Treg cells. The iTreg cells were generated by culturing Treg-cell-depleted naïve $CD4^+$ T cells for 6 days in the presence or absence of P4 (2 μg/mL or 6.4 μM) and TGF-β1. Concanavalin A (2.5 μg/mL) and IL-2 (100 U/mL) were used for T-cell activation. Data shown are representative of three (A) and two (B) independent experiments.

suppression of the mTOR pathway. Compared to WT T cells, PR (−/−) T cells were less affected by P4 in the S6 protein phosphorylation (Fig. 5B), suggesting a role for PR in this suppression. The suppressive effect of P4 on S6 protein phosphorylation was detected even at an early time point (60 min) during T-cell activation and in the presence of inhibitors of transcription or translation (Supporting Information Fig. 2). Thus, the nongenomic function of P4 appears to be involved in this suppression. mTORC1 kinase is activated by the GTPase Ras homolog enriched in brain (Rheb) [39]. Using a retroviral expression method, we overexpressed Rheb to increase the activation status of the mTOR pathway. This Rheb overexpression decreased the activity of P4 in generation of LAP-TGF-$\beta1^+$ iTreg cells (Fig. 5C). We examined LAP-TGF-β1 instead of FoxP3 expression because GFP expression, which identifies cells overexpressing RheB, becomes undetectable if the cells are fixed, and permeabilized for FoxP3 detection. These results indicate that increased activity of the mTOR pathway can counteract the activity of P4 on T-cell differentiation. However, the suppression was partial indicating also the possibility of mTOR/Rheb-independent regulation of T cells.

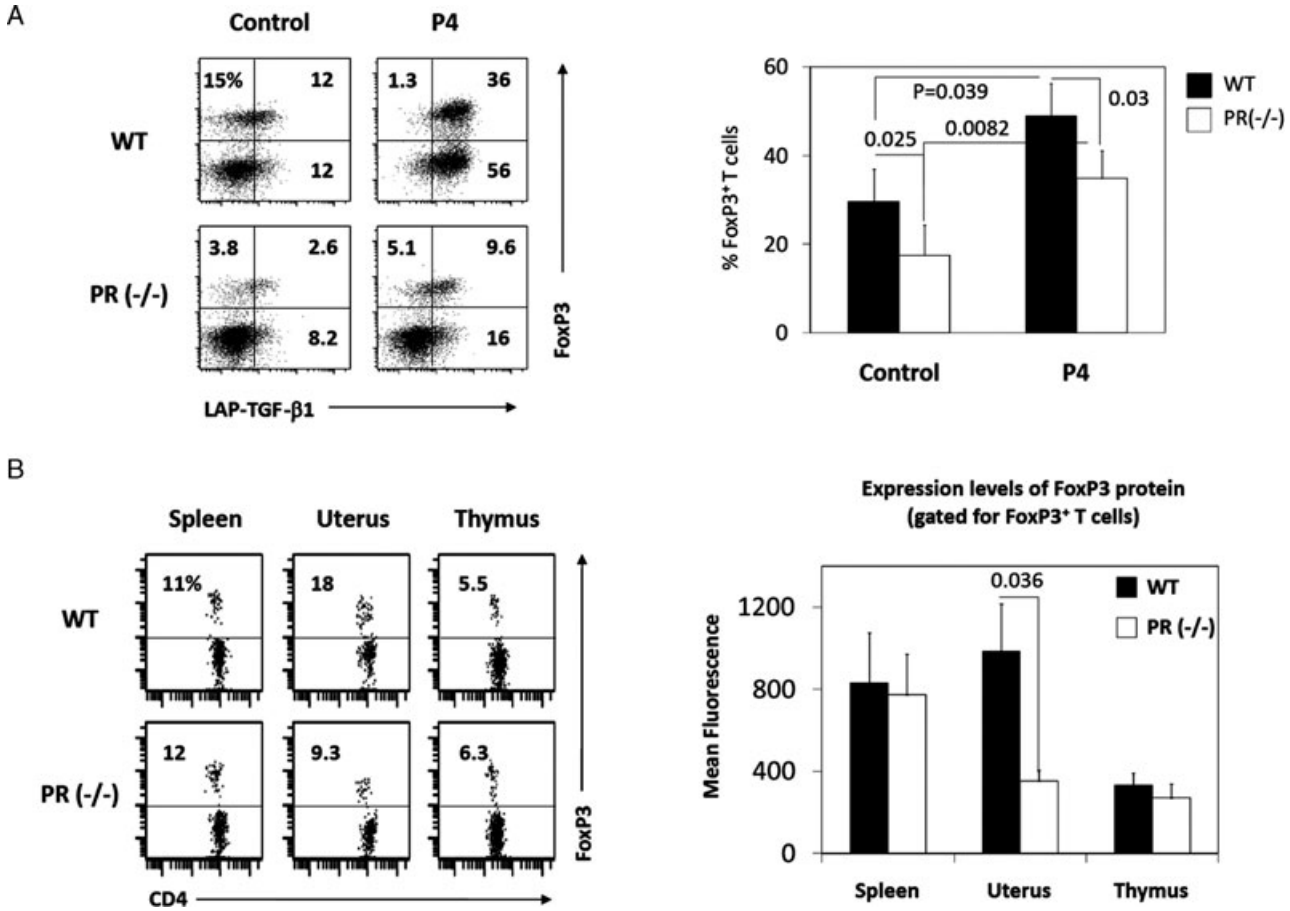

Figure 3. P4-induced FoxP3 expression is partially dependent on nuclear progesterone receptor. (A) Responses of PR (−/−) T cells to P4 in differentiation into iTreg cells. Cells were cultured as in Fig. 1D and stained for LAP-TGF-β1 and FoxP3 expression. (B) Frequencies of FoxP3$^+$ T cells in the uterus, spleen, and thymus of PR (−/−) mice. Data for CD4$^+$CD8$^-$ T cells are shown for the thymus. Decreased expression of FoxP3 by CD4$^+$ T cells in the uterus of PR (−/−) mice is shown in the graph (right). Mean fluorescence intensity indicates FoxP3 expression levels in the T cells of wild-type and PR (−/−) mice (right). Combined data are shown as mean + SEM of 4–6 samples pooled from four independent experiments and flow cytometry plots are representative. Significant differences ($p < 0.05$) between two groups determined by Student's *t*-test are shown.

P4-induced FoxP3$^+$ T cells display improved suppressive activities in vivo

To assess the function of P4-iTreg cells in regulation of inflammatory diseases in vivo, we prepared MOG-specific P4-iTreg cells (induced with IL-2, TGF-β1, and P4) and control iTreg cells (induced with IL-2 and TGF-β1) from naïve CD4$^+$ T cells prepared from MOG-specific 2D2 transgenic mice, and compared their regulatory activities in vivo. As shown in Supporting Information Fig. 3A, only the P4-induced Treg group had high frequencies of FoxP3$^+$ T cells after the two rounds of culture. We examined their regulatory activities on experimental allergic encephalomyelitis (EAE) induced in the central nervous system by a MOG peptide. P4-iTreg cells were superior to control-iTreg cells in suppressing antigen-induced EAE (Fig. 6A and B). P4-iTreg cells were more efficient than control iTreg cells in decreasing infiltration of the spinal cord with inflammatory cells (Fig. 6C). Additionally, we examined if the P4-iTreg cells can suppress the emergence of effector T cells (Th1 and Th17 cells) at an early time point after onset of the disease. We found that P4-iTreg cells were able to decrease the numbers of Th17 and Th1 cells in the CNS (Fig. 6D). Th17, but not Th1, cell frequencies were decreased in the CNS tissues (Supporting Information Fig. 3B). P4-iTreg cells were statistically more efficient than control iTreg cells in suppression of the inflammatory cells in the brain.

We further investigated if the P4-iTreg cells can control inflammation in a different organ (the intestine). We utilized a naïve T-cell-induced colitis model in Rag1-deficient mice. We found that P4-iTreg cells were more efficient than the control iTreg cells in preventing colitis as evidenced by decreased weight loss (Fig. 7A), numbers of IFN-γ$^+$ T cells (Fig. 7B), and tissue inflammation indicated by mucosa hyperplasia in the distal colon (Fig. 7C). When compared to CD4$^+$CD25$^+$ nTreg cells, P4-iTreg cells were somewhat more efficient in suppression of colitis and inflammatory T cells (Supporting Information Fig. 4A and B). The frequencies of the two Treg populations were similar in various tissues (Supporting Information Fig. 4C). Overall, the results demonstrate that P4 induces iTreg cells that are highly suppressive in vivo.

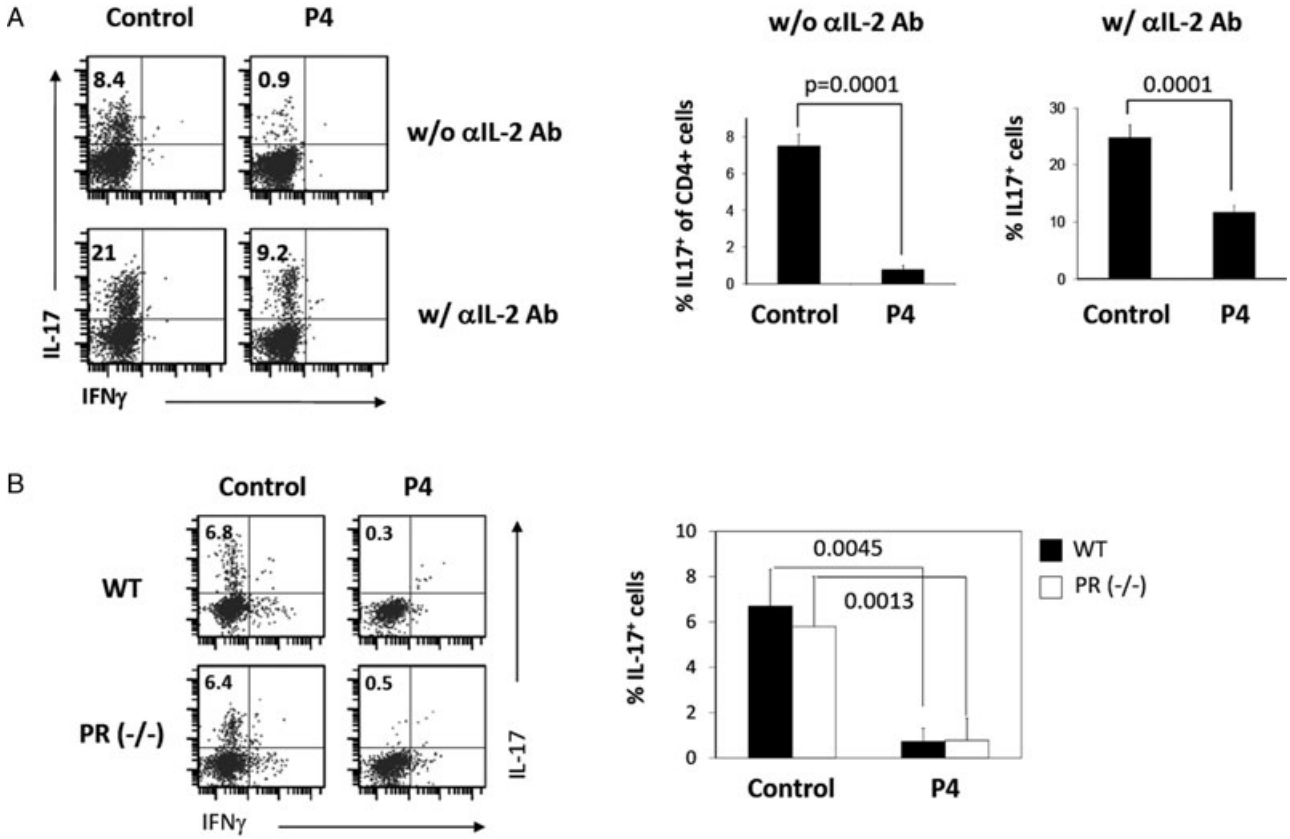

Figure 4. P4 suppresses Th17-cell induction. (A) P4 effects on Th17-cell induction. Treg-cell-depleted naïve $CD4^+$ T cells, isolated from lymph nodes and spleen, were cultured for 6–7 days under Th17-polarizing conditions (anti-IFN-γ, anti-IL-4, IL-6, TGF-β1, and concanavalin (A) in the presence or absence of P4 (2 μg/mL or 6.4 μM). (B) Naïve $CD4^+$ T cells isolated from WT and PR (−/−) mice were cultured as in (A) and examined for induction of Th17 cells. Combined data are shown as mean + SEM of 4–8 sampled pooled from 4–8 independent experiments; flow cytometry plots are representative. Significant differences ($p < 0.05$) between two groups determined by Student's *t*-test are shown.

The P4-induced iTreg population is more stable than control iTreg cells in expression of FoxP3

Treg cells induced in vitro are unstable in expression of FoxP3 and readily revert back to non-Treg cells [15]. Because of the high induction rate of FoxP3 in T cells in response to P4 and of the highly suppressive function of P4-induced Treg cells in vitro and in vivo, we examined if P4 stabilizes the FoxP3 expression in the T-cell population. For this, we recultured the control iTreg cells and P4-iTreg cells in the presence or absence of P4 and assessed the expression of FoxP3 (Fig. 8A). We intentionally used a high concentration of TGF-β1 to prepare iTreg cells enriched with $FoxP3^+$ T cells in this experiment. Upon reculture, 70% of the cells in the control iTreg population lost the expression of FoxP3. However, only ∼30% of the cells in the P4-iTreg population lost the expression of FoxP3. Only ∼20% of the cells in the P4-iTreg population lost the FoxP3 expression when they were cultured in the presence of P4. In contrast, ∼50% of the cells in the control iTreg population lost the FoxP3 expression. These results are supported well by the data in Supporting Information Fig. 3A. The P4-iTreg cells were highly stable also in vivo in inflammatory conditions. Compared to control iTreg cells, almost all of which reverted back to $FoxP3^-$ cells, a significant portion of P4-iTreg cells in the spleen and the draining lymph node remained as $FoxP3^+$ T cells even 4 weeks after the cell transfer in MOG-induced EAE mice (Fig. 8B) and T-cell-induced colitis mice in Rag1-deficient mice (Supporting Information Fig. 5). These results indicate that P4 makes iTreg cells with improved stability.

Discussion

P4, a major female sex hormone that promotes and maintains pregnancy, has potent immune–regulatory functions. P4 steers T helper-cell differentiation into Treg cells but suppresses the generation of effector T helper cells [24, 25]. This function of P4 could be utilized to generate iTreg cells to control inflammatory diseases through generation of highly suppressive iTreg cells ex vivo. Direct administration of P4 into patients as a hormone immunotherapy is a possibility but significant off-target side effects are expected. Low stability of iTreg cells is a major concern in utilizing these

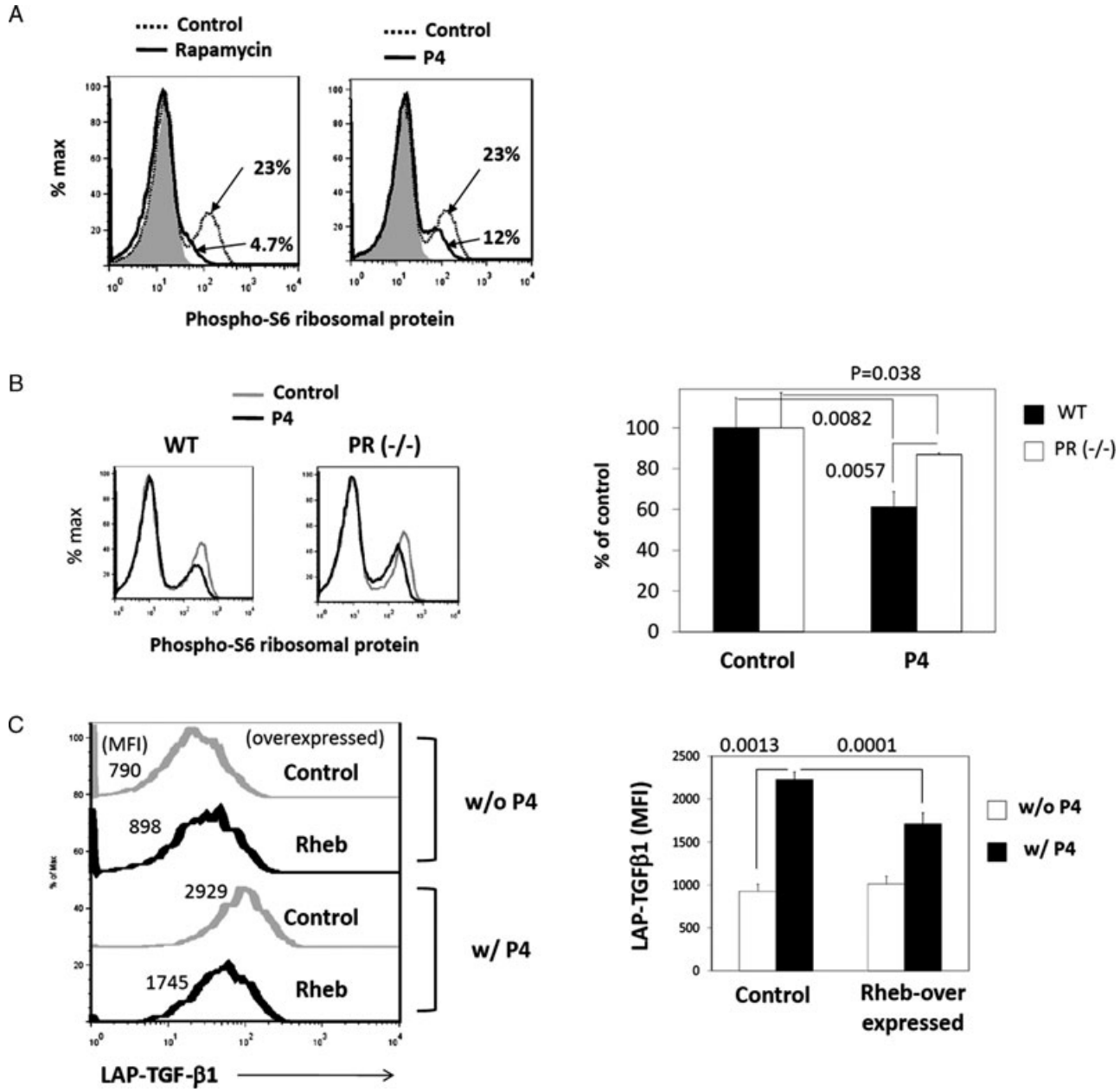

Figure 5. Activation of the mTOR pathway is suppressed by P4. (A) Rapamycin (3 nM) or P4 (2 μg/mL or 6.4 μM) was examined for their effects on phosphorylation of ribosomal S6 protein (Ser235/236) in antigen-primed T cells. Treg-cell-depleted naïve $CD4^+$ T cells, isolated from lymph nodes and spleen, were activated for 2 h with anti-CD3/28 beads and IL-2 (200 U/mL) in the presence of rapamycin or P4. Staining with an isotype control antibody is shown in the gray-filled histogram. (B) S6 protein phosphorylation in PR (−/−) T cells. The data are shown as % of the control groups without P4. (C) Rheb was overexpressed in Treg-cell-depleted naïve $CD4^+$ T cells by a retroviral gene transfer method and cultured for 5 days. P4 was added to the culture as indicated, and T-cell differentiation into LAP-TGF-$\beta1^+$ Treg cells was examined. Combined data are shown as mean + SEM of 3–8 samples pooled from three independent experiments; qualitative data are representative. Significant differences ($p < 0.05$) between two groups determined by Student's *t*-test are shown.

cells in clinical applications. The questions that we had regarding the induction of iTreg cells by P4 included: (i) can the P4-induced iTreg cells be utilized to suppress inflammatory diseases in vivo? (ii) Are classical P4 nuclear receptors involved in P4-mediated generation of iTreg cells? (iii) What is the intracellular signaling process affected by P4 in T cells? Our results provided insights into these issues.

Our results indicate that iTreg cells induced by P4 are highly effective in suppressing inflammatory diseases. We used an EAE model and an inflammatory bowel disease (IBD) model. In both models, the iTreg cells induced by P4 were significantly better than control iTreg cells induced by TGF-β1 alone. iTreg cells appear to have variable activities in suppression of tissue inflammation [17–20]. Our results showed that regular (control)

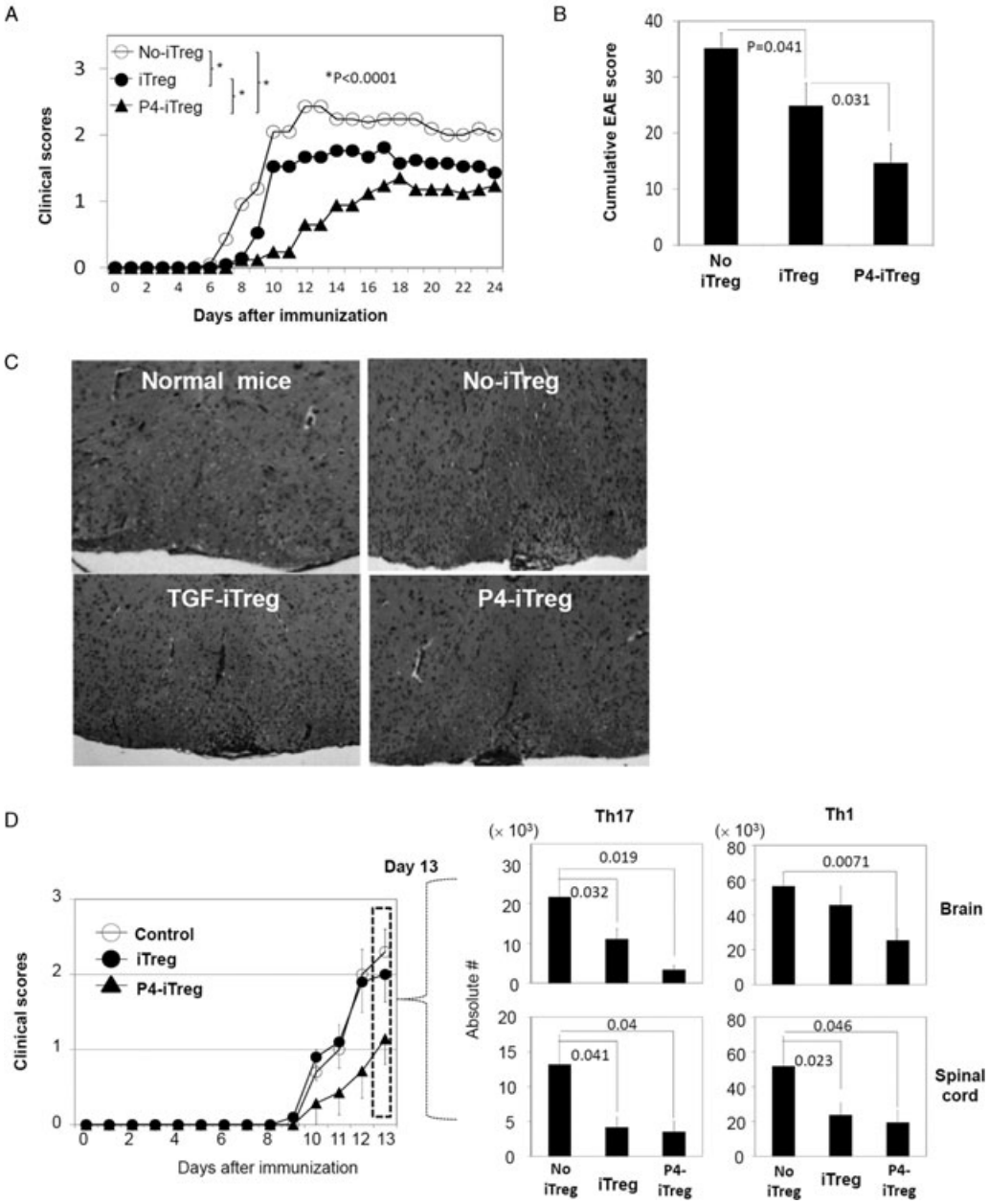

Figure 6. P4-induced iTreg cells are more effective than iTreg cells in suppressing EAE development. (A) In vivo regulatory function of P4-induced antigen (MOG)-specific Treg cells in regulation of EAE ($n = 17–21$/group). (B) Cumulative clinical scores are the sums of all EAE clinical scores divided by the number of mice per group. Data are shown as mean + SEM of 17–21 mice per group and are pooled from two independent experiments. (C) H&E histology of the spinal cords of the EAE mice treated with no Treg cells, control (TGF-β1), or P4-iTreg cells. A representative image ($n = 17–21$/group; ×100 original magnification) from healthy unimmunized mice without inflammation is also shown as a negative control. (D) P4-iTreg cells are effective in suppression of emergence of inflammatory T cells at an early time point (day 13). Absolute numbers of effector T cells from each tissue/organ are shown as mean + SEM of 17–21 samples per group pooled from two independent experiments. The iTreg cells were prepared with a MOG peptide and irradiated splenocytes from 2D2 mice (A) or MOG-immunized mice (D) as described in *Materials and Methods*. Significant differences ($p < 0.05$) between two groups determined by repeated measures ANOVA (A) or Student's *t*-test are shown (B, D).

iTreg cells have significant suppressive effects on EAE development. Although significant, the level of suppression was not high, and very few Treg cells remained positive for FoxP3 in the EAE mice injected with iTreg cells. In contrast, P4-induced iTreg cells were effective in suppressing EAE and many more P4-iTreg cells than control iTreg cells remained positive for FoxP3. Control iTreg cells also had a suppressive activity on the development of colitis at a moderate level. Compared to the control iTreg cells, P4-iTreg cells were more efficient in suppression of IBD. The superior in vivo suppressive functions are associated with two features of P4-induced iTreg cells. P4-induced iTreg cells are more stable and better sustain as FoxP3$^+$ T cells than control iTreg cells. Another

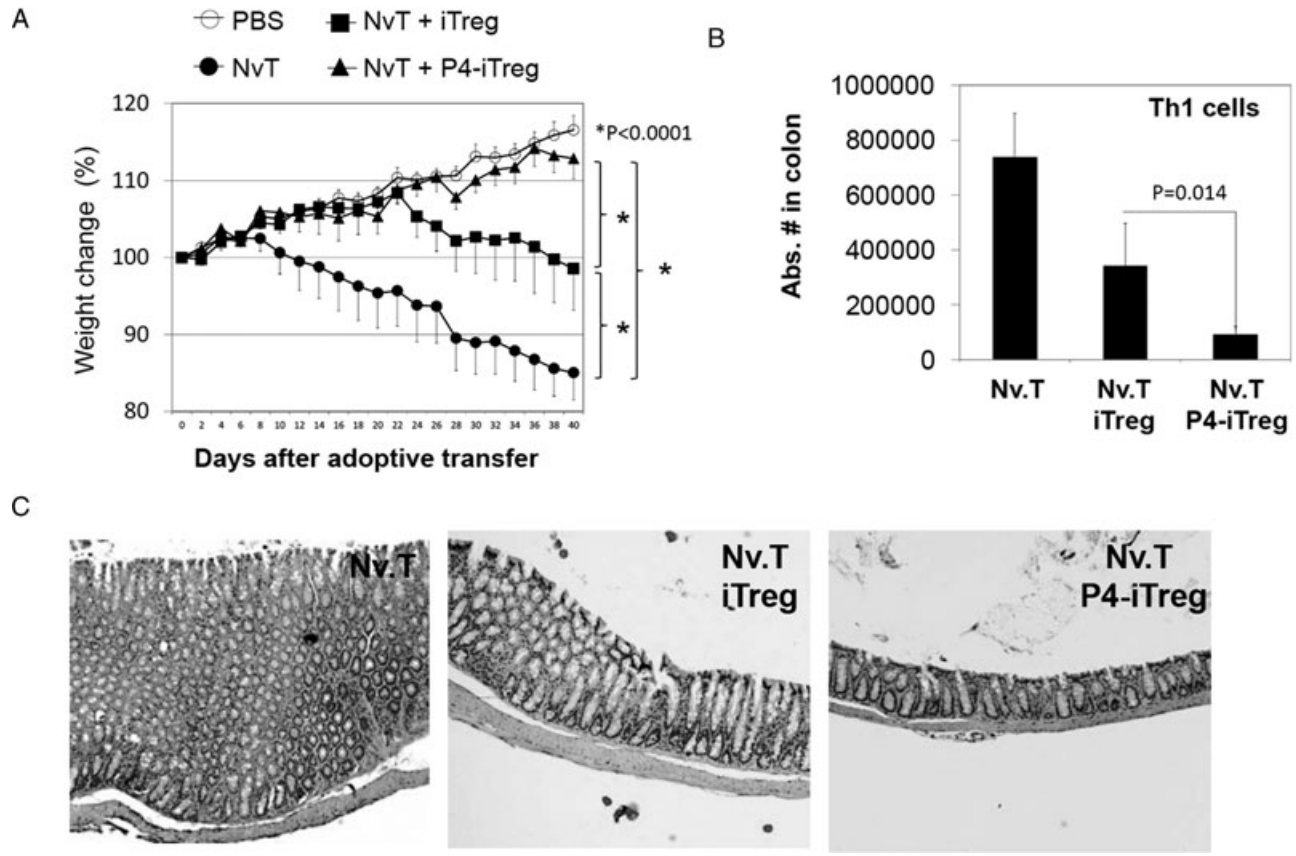

Figure 7. P4-induced iTreg cells are more effective than control iTreg cells in suppressing murine colitis. (A) P4-iTreg cells were effective in preventing inflammatory bowel disease in Rag1-deficient mice. iTreg cells were prepared as described for Fig. 1D and injected into Rag1-deficient mice to suppress T-cell-induced colitis development. Weight change due to inflammation is shown as mean + SEM of $n = 11–15$/group. Data from two independent experiments were pooled. (B) Absolute numbers of Th1 cells in the distal colon of Rag1-deficient mice injected with naïve T (Nv.T) cells and indicated Treg cells were determined. Data are shown as mean + SEM of $n = 6–8$ and are representative of/pooled from two different experiments performed. (C) Representative hematoxylin and eosin staining of the distal colon of the Rag1-deficient mice (×100 original magnification). Data shown are representative of two experiments performed. Significant differences ($p < 0.05$) between two groups determined by one-way analysis of variance (A) or Student's *t*-test are shown (B).

feature that can help them function better as Treg cells in vivo would be high expression of LAP-TGF-β1, which is the major suppressive cytokine produced by Treg cells [40]. We, however, did not demonstrate that this LAP-TGF-β1 is required for their suppressive functions. An added function of P4 in suppression of inflammatory diseases is inhibition of Th17 cells. P4 promotes the generation of iTreg cells at the expense of potentially inflammatory Th17 cells. This selective promotion of Treg cells over Th17 cells would have implications in establishing immune tolerance.

We found that nuclear P4 receptors are required for the optimal effect of P4 on T-cell differentiation. We observed the PR A/B (−/−) naïve T cells, while they are still able to respond to P4 at low rates, were less efficient in becoming iTreg cells in response to P4. This residual effect of P4 could be mediated through other non-nuclear P4 receptors. PR A/B (−/−) naïve T cells were less responsive to TGF-β1 plus IL-2 than WT cells even in the absence of exogenous P4. This difference could be due to the fact that fetal bovine serum usually contains high levels of P4. We used charcoal-treated fetal bovine serum but this treatment would not completely remove P4 from the culture. Our results suggest that PR is partially responsible for the suppressive P4 effect on mTOR signaling and induction of Treg cells. However, PR appears to have no detectable role in suppression of Th17 cells by P4 at least in vitro.

Blood progesterone levels at follicular phase in humans is low (1–2 nM) but can reach up to 500 nM in pregnancy [41]. P4 concentration is estimated to be ~3 μg/g of placenta tissue. Induction of Treg cells was detectable at 0.5 μg/mL (1.6 μM) of P4 and was maximal at 2 μg/mL (6.4 μM) in our study. Thus, the concentration (2 μg/mL) that we used for most experiments is close to the P4 concentration in placenta and potentially other tissues in pregnancy. The focus of this study is to generate iTreg cells for therapeutic purposes, and we do not claim that the induction occurs in vivo. This, however, is highly plausible and would be a topic of interest for future studies.

Effective concentrations of P4 to induce mouse and human Treg cells are similar [24]. One apparent difference between the two species was that human adult peripheral blood naïve T cells were not amenable to make FoxP3$^+$ T cells in response to P4 unlike their cord blood counterparts, whereas adult naïve T cells

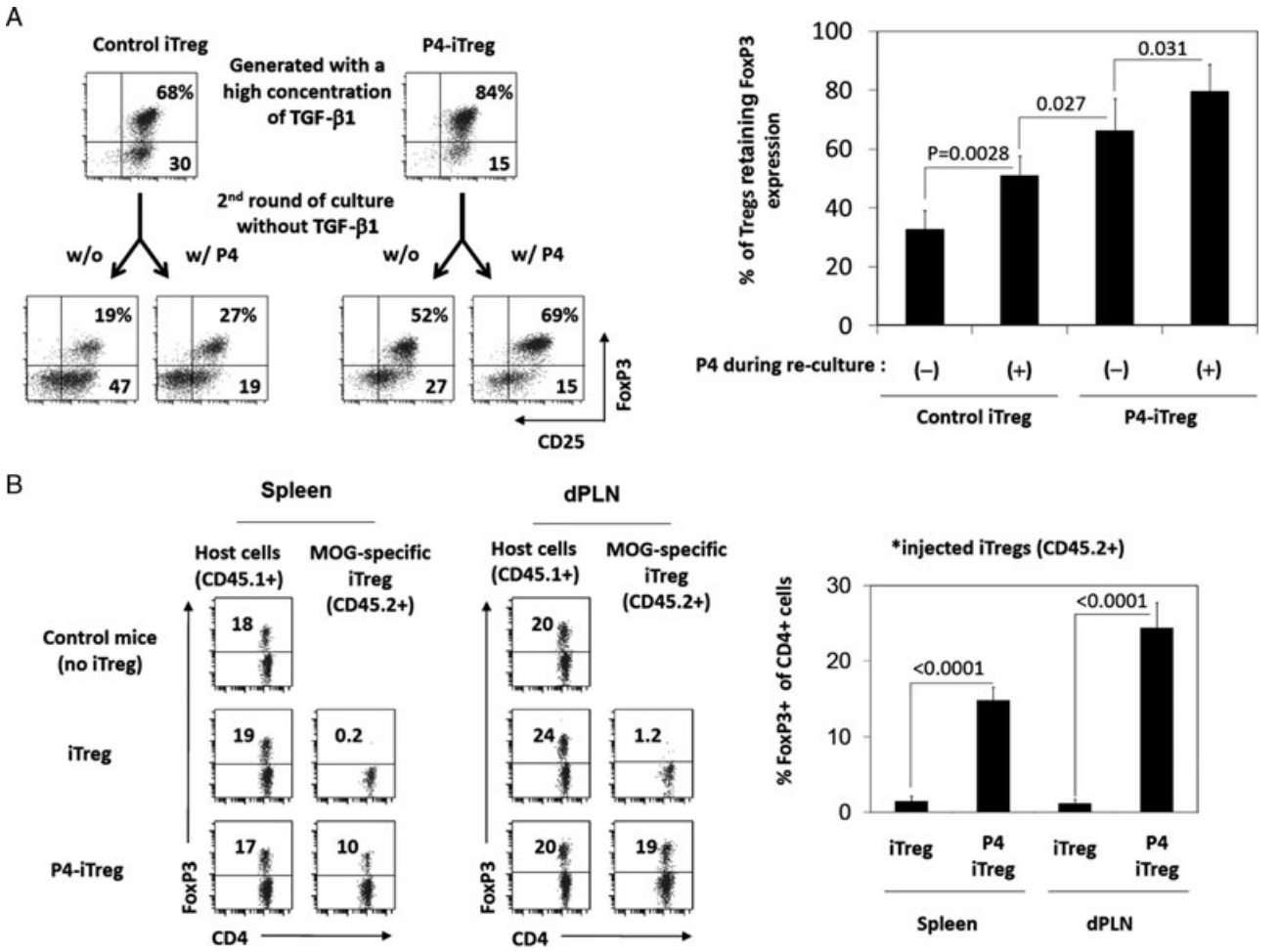

Figure 8. P4-induced iTreg cells are more stable than control iTreg cells in FoxP3 expression. (A) Treg-cell stability in vitro. Treg-cell-depleted naïve CD4$^+$ T cells were cultured for 6–7 days with concanavalin A in the presence of TGF-β1 (2 ng/mL) for control-iTreg cells or with TGF-β1 and P4 (2 μg/mL or 6.4 μM) for P4-iTreg cells. The T cells were recultured in the absence or presence of P4 and examined for FoxP3 expression. Quantitative data are shown as mean + SEM of five samples pooled from five independent experiments; flow cytometry data are representative. (B) Treg-cell stability in vivo in an inflammatory condition. Frequencies of iTreg cells in the spleen and draining lymph nodes of EAE mice 28 days after the cell transfer are shown. A representative set of dot plot data and mean + SEM of $n = 9$–10 pooled from two independent experiments are shown. Significant differences ($p < 0.05$) between two groups determined by Student's t-test are shown.

from mice were more readily converted into FoxP3$^+$ T cells in response to P4. One should note that the tissue sources (lymphoid cells versus peripheral blood cells) for the T cells are different in addition to the difference in species.

Effect of P4 on gene expression is complex and involves direct DNA binding and nonbinding functions of PR [42]. This is further complicated by the roles of non-nuclear PR receptors in mediating the P4 effect on T cells. Thus, it is difficult to pinpoint a single major target of the P4 effect in T cells. Despite this issue, we observed that the mTOR pathway is negatively regulated by P4. The mTOR pathway is composed of mTORC1 and mTORC2 [43, 44]. The major function of mTORC1 is to activate p70 S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) for translation regulation [43, 44]. Rheb is a key molecule upstream of mTORC1 and activates mTORC1. In T helper cells, mTORC1 is required for generation of Th1 and Th17 cells, and its suppression enhances the generation of Treg cells [35, 36]. Our data indicate that P4 suppresses the mTORC1 signaling, and this is associated with enhanced generation of iTreg cells with P4. Moreover, we were able to demonstrate that enforced expression of Rheb decreased the function of P4 in skewing T-cell differentiation.

Our results provide not only insights into the role of P4 in regulation of T-cell function but also a novel method to generate iTreg cells with improved stability and efficacy in suppression of tissue inflammation. The results also provided information regarding the receptors involved in P4 function in regulation of CD4$^+$ T cells and identified a relevant signaling pathway regulated by P4. These findings support the role of P4 in promoting immune tolerance and identify the utility of P4 in generating iTreg cells with high potentials in treating inflammatory diseases.

Materials and methods

Animals, cells, and reagents

PR (−/−) mice in the C57BL/6-CBA mixed or C57BL/6 background were described before [45]. Rag1-deficient mice in the C57BL/6 background (B6.129s7-Rag1^{tm1Mom}/J), 2D2 transgenic mice (C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J), and CD45.1 mice (B6.SJL-Ptprca Pepcb/BoyJ) were obtained from the Jackson Laboratory (Bar Harbor, Maine). DO11.10 Rag2 (−/−) mice were purchased from Taconic Farms (Hudson, NY). These mice were housed in a specific pathogen-free condition at Purdue and used at 6–8 weeks of age for most experiments. The responses of T cells from male and female mice were similar, and thus the mice were used without discrimination based on sex (Supporting Information Fig. 1). CD4$^+$ T cells were isolated from mouse total lymphocytes by a negative selection method (CD4$^+$ T Cell Isolation Kit II, Miltenyi Biotec Inc, Auburn, CA). Then, naïve T cells (∼95% pure) were isolated using antibodies to CD44 (IM7), CD25 (PC61), CD69 (H1.2F3), CD8 (53.6.7), and CD19 (6D5) to further deplete activated, Treg, memory, and contaminating non-CD4$^+$ T cells. The antibodies were purchased from BioLegend (San Diego, CA). For preparation of nTreg cells (∼70% were FoxP3$^+$ cells), CD4$^+$CD25$^+$ cells were positively selected using an antimouse CD25 antibody (PC61) among the total CD4$^+$ T cells.

Indicated tissues were harvested from 2- to 4-month-old female WT or PR (−/−) mice. The tissues were digested with RPMI medium containing collagenase type 3 (2 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 1 h at 37°C for characterization of T cells in tissues. The cells were stained with antibodies to CD4 (RM4-5), CD103 (2E7), and FoxP3 (FJK-16S, eBioscience, San Diego, CA). For intracellular staining, cells stained with antibodies to CD4 and CD44 were activated for 4 h with PMA, ionomycin, and monensin for intracellular staining with antibodies to IL-17 (TC11-18H10.1) and IFN-γ (XMG1.2). P4 (4-Pregnene-3,20-dione, catalog # P8783, Sigma-Aldrich, St. Louis, MO) was prepared in absolute ethanol (5 mg/mL) and used at indicated concentrations.

In vitro T-cell differentiation in response to P4

Naïve mouse CD4$^+$ T cells, isolated from WT, CD45.1 congenic, PR (−/−) mice, DO11.10 Rag2 (−/−) mice, or 2D2 transgenic mice, were cultured for 6–7 days with concanavalin A (Sigma-Aldrich, St. Louis, MO) (2.5 μg/mL), $OVA_{323-339}$ peptide (1 μg/mL), MOG antigen peptide (MOG_{35-55}, AnaSpec (Fremont, CA), 10 μg/ml), or anti-CD3/CD28 antibodies (plate-bound anti-CD3 at 1 μg/mL, clone 145-2C11; soluble anti-CD28 at final 0.5 μg/mL, clone 37.51, all from BioXCell (West Lebanon, NH)) in the presence or absence of P4 in RPMI medium. IL-2 (100 U/mL) and TGF-β1 (PeproTech, Rocky Hill, NJ) were added to induce Treg cells. For activation with MOG_{35-55}, irradiated mouse splenocytes were used at three times of the number of T cells. Because the results were similar with all of the T-cell activators, concanavalin A was used consistently for experiments described in other figures. MOG_{35-55} was used in Fig. 6. For Th17 cell induction in the presence of P4, naïve T cells were cultured in a cocktail of reagents (anti-IFN-γ, anti-IL-4, IL-6, and TGF-β1; from BioLegend, San Diego, CA) and cultured for 6–7 days as described previously [46]. Expression of FoxP3, LAP-TGF-β1 (TW7-16B4), or IL-17/IFN-γ (BD Bioscience, San Jose, CA) was determined by a Canto II flow cytometer (BD Biosciences).

Methylation status of TSDR

Genomic DNA was isolated from naïve T cells, iTreg cells, or nTreg cells using QIAamp DNA kits (Qiagen, Valencia, CA). The isolated DNA was modified by sodium bisulfite using the CpGenome DNA modification kit (EMD Millipore, Billerica, MA). PCR was performed as described [15] and amplified TSDR fragment was sequenced. The sequencing data were visualized by the FinchTV software (PerkinElmer, Waltham, MA) to assess degree of methylation.

Measurement of the in vitro suppressive activity

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4$^+$CD25$^-$ T cells (target cells, 3×10^4 cells/well) and iTreg cells as suppressors were cocultured in round-bottom 96-well plates for 3 days at indicated ratios with anti-CD3 antibody (2 μg/mL) and irradiated splenic APCs (9×10^4 cells/well). iTreg cells were prepared by activation of naïve T cells with concanavalin A (2.5 μg/mL) and IL-2 (100 U/mL) for 5 days. P4 (2 μg/mL or 6.4 μM) and/or TGF-β1 (1 ng/mL) were added as indicated. Dilution of CFSE was determined by flow cytometry.

Assessment of the in vivo stability and regulatory activity of P4-induced FoxP3$^+$ T cells

For the MOG peptide-induced EAE model, 5×10^6 P4-induced or control iTreg cells were injected i.v. into CD45.1$^+$ congenic C57BL/6 mice and the mice were immunized with MOG_{35-55} peptide (100 μg/mouse) in complete Freund's adjuvant. On the same day, pertussis toxin (100 ng/mouse, PTX from List Biological Laboratories (Campbell, CA)) was injected i.p. The immunization was repeated 7 days later. The control iTreg cells (containing iFoxP3$^+$ T cells) were prepared ex vivo by culturing naïve CD4$^+$ T cells isolated from lymph node cells and splenocytes of 2D2 mice (Figs. 6A and 8B) or total CD4$^+$ T cells from C57BL/6 mice immunized with MOG_{35-55} peptide 7 days prior to sacrifice (Fig. 6D) in the presence of MOG_{35-55} peptide (10 μg/mL), TGF-β1 (0.5 ng/mL), and IL-2 (25 U/mL). Irradiated splenocytes

were added to activate the T cells for 6 days. P4 (2 μg/mL or 6.4 μM) was used to make P4-induced Treg cells. This culture was repeated again. Scoring was performed as followings: normal mouse; no overt signs of disease (0); limp tail or hind limb weakness but not both (1); limp tail and hind limb weakness (2); partial hind limb paralysis (3); complete hind limb paralysis (4); moribund state; and death by EAE (5).

For the naïve T-cell-induced colitis model in Rag1-deficient mice, naïve CD45.1$^+$ CD4$^+$ T cells (3×10^5/mouse) were injected i.p. together with CD45.2$^+$ iTreg or nTreg cells (6×10^5/mouse) into Rag1-deficient mice. Mice were monitored for weight change and sacrificed 30 days later. The use of these congenic mice allowed us to separately identify the T cells derived from the iTreg cells versus naïve T cells. A hematoxylin and eosin (H&E) staining was performed on 6 μm paraffin tissue sections to assess histological changes.

Assessment of in vitro stability of iTreg cells induced by P4

Naïve T cells were activated with concanavalin A (2.5 μg/mL), IL-2 (100 U/mL), and TGF-β1 (2 ng/mL) for 5–6 days in the presence of P4. The iTreg cells were recultured with concanavalin A (2.5 μg/mL) or anti-CD3/CD28 beads (Miltenyi Biotec, Auburn, CA) and IL-2 (100 U/mL) in the presence or absence of TGF-β1, or P4 for 5–6 days. FoxP3 expression was determined by flow cytometry.

Assessment of activity of the mTOR pathway

Naïve CD4$^+$ T cells were isolated from C57BL/6 mice and stimulated with anti-CD3/CD28 beads (Miltenyi Biotec, Auburn, CA) and IL-2 (200 U/mL) in the presence of P4 for 2 h. T cells were pretreated with actinomycin D (AMD; 5 μg/mL) or cycloheximide (CHX; 10 μg/mL) for 30 min prior to the activation and further treated with the same inhibitor during the activation for 60 min with or without P4. AMD and CHX are from Sigma-Aldrich (St. Louis, MO). Activated cells were fixed 1% paraformaldehyde for 30 min and permeabilized in BD Phosflow Perm buffer III (BD bioscience) for overnight. Then, cells were stained with anti-phospho S6 ribosomal protein antibody (D57.2.2E, Cell Signaling, Danvers, MA) and antimouse CD4 antibody for 30 min at room temperature before flow cytometric analysis

Retroviral overexpression of Rheb

Murine Rheb was cloned into a LZRS-pBMN-IRES-GFP vector as described previously [47]. Viral supernatant was prepared by Rheb transfection into Phoenix eco cells. For viral infection, naïve cells were activated for overnight with concanavalin A (2.5 μg/mL) and IL-2 (100 U/mL). Activated cells were infected with virus-containing conditioned medium in the presence of polybrene (6 μg/mL) by centrifugation at 1200*g* for 90 min at 30°C. Cells were further cultured in a Treg condition for 5 days in the presence of P4 (2 μg/mL) as described above.

Statistical analysis

Student's *t*-test (for most figures) and one-way analysis of variance (ANOVA) were used with SAS 9.2 or Prism (GraphPad Software) to determine significance of the differences between groups. p values ≤ 0.05 were considered significant. All error bars shown in this paper are SEM.

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Conflict of interest: Some of the findings in this manuscript are included in our US patent application (65635.P1.US).

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Abbreviations: **LAP**: latency-associated peptide · **mTOR**: mammalian target of rapamycin · **P4-iTreg cell**: progesterone-induced Treg cell · **P4**: progesterone · **PR**: progesterone receptor · **Rheb**: Ras homolog enriched in brain · **TSDR**: Treg-specific demethylated region

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