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

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Summary

While induced FoxP3⁺ T cells (iTregs) are promising cellular therapeutics to treat inflammatory diseases, a limitation in utilizing iTregs 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 iTregs with highly suppressive activity and improved stability in vivo. We generated iTregs with progesterone in vitro and found that progesterone generates iTregs that are highly stable in inflammatory conditions. Moreover, P4-induced iTregs highly express latency-associated peptide TGFβ1 and are efficient in regulating inflammation in multiple tissues, whereas control iTregs induced with TGFβ1 alone are less stable and ineffective in suppressing inflammation. The function of progesterone in inducing iTregs 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 non-nuclear progesterone receptors are involved in mediating the function. We conclude that P4 can be utilized to generate iTregs with a high therapeutic potential in treatment of tissue inflammation.

Keywords

FoxP3; progesterone; inflammation

Introduction

FoxP3⁺ T cells (commonly called Tregs) are a specialized subset of CD4⁺ T cells with immune suppressive functions [1, 2]. Tregs can be made in the thymus from T cell progenitors as natural Tregs (nTregs) and from naïve T cells as induced Tregs (iTregs) in the periphery. iTregs are induced in the periphery in response to various signals including TCR activation, cytokines (IL2 and TGFβ1), nuclear hormone receptor ligands, and other tissue factors [3–6]. Tregs produce TGFβ1, IL10 and/or IL35, 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 IFNγ, IL4, and/or IL17. Among the effector T cells, IL17-producing T cells (commonly called Th17 cells), while related to

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Conflict of interest.

Some of the findings are included in our US patent application (65635.P1.US).

Tregs in development in that they are induced by TGF β 1, mount immune responses mainly for immunity and inflammation [10–12].

Because of their suppressive activity on many types of immune cells, Tregs are considered potential therapeutics to treat inflammatory diseases. Compared to nTregs, iTregs that are generated in vitro are less stable and readily become effector T cells [13, 14]. In this regard, there is a difference between nTregs and iTregs in methylation of certain CpG motifs in the Foxp3 locus [15, 16]. nTregs, while more stable and effective in suppression, are difficult to prepare in sufficient numbers for therapeutic applications. Some reported that iTregs induced in vitro fail to control tissue inflammation [17, 18], whereas others reported significant suppression [19, 20]. It is highly desirable to generate iTregs 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 iTregs from human cord blood naive T cells [24, 25]. We investigated if P4 would be used as an iTreg-inducing agent to prepare iTregs with improved stability and therapeutic property. Our study revealed that P4 can be used to generate iTregs with improved stability in inflammatory conditions and more effectively control inflammatory diseases in the central nervous system and the intestine than control iTregs induced with TGF β 1 alone.

Results

P4 induces LAP-TGF β 1-expressing FoxP3⁺ T cells

While the tolerogenic effect of P4 in expanding murine Tregs in pregnancy has been reported [25], the induction from naïve T cells, phenotype, and in vivo function of mouse iTregs induced by P4 have not been determined. First, we examined the function of P4 in control of naïve mouse T cell differentiation into iTregs. P4 increased the differentiation of naïve T cells into FoxP3⁺ T cells (Fig. 1A). This process was greatly enhanced in the presence of TGF β 1. We compared multiple T cell activators such as concanavalin A (Fig. 1A), anti-CD3/CD28 (Fig. 1B), and OVA_{323–339} 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_{35–55}; not shown). Naïve CD4⁺ T cells from both male and female mice were equally responsive in becoming FoxP3⁺ T cells in response to P4 (Supplementary Fig. 1). P4 increased the frequency and absolute numbers of FoxP3⁺ T cells in a dose-dependent manner in the presence of exogenous TGF β 1 (Fig. 1C). The iTregs induced in the presence of P4 (P4-iTregs) highly expressed latency-associated peptide (LAP)-TGF β 1 (Fig. 1D), which is the inactive precursor form of active TGF β 1.

P4-iTregs were highly efficient in suppressing the proliferation of target T cells (Fig. 2A). P4-iTregs were more suppressive than control iTregs induced in the absence of exogenous P4. Even P4-iTregs induced in the absence of exogenous TGF β 1 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 nTregs but not in iTregs [15]. We examined if P4 has any effect on the methylation status of TSDR. The TSDR of P4-iTregs was largely methylated and similar to control iTregs (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 (mPR α , mPR β , and mPR γ), 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 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 iTregs, 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 TGFβ1, factors that promote Tregs 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 IL17⁺ CD4⁺ T cells. We found that P4 dampens mouse T cell differentiation into Th17 cells driven by TGFβ1 and IL6 (Fig. 4A). This occurred also with anti-IL2 that neutralizes IL2, a cytokine which is known to suppress 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 Tregs [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 which is a downstream target of the mTORC1 kinase. 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 function of suppressing the mTOR pathway is consistent with the Treg-inducing activity of P4. This data suggests a possibility that P4 can induce Tregs through suppression of the mTOR pathway. 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 (Supplementary Fig. 2). Thus, the non-genomic 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 over-expressed Rheb to increase the activation status of the mTOR pathway. This Rheb over-expression decreased the activity of P4 in generation of LAP-TGFβ1⁺ iTregs (Fig. 5C). We examined LAP-TGFβ1 instead of FoxP3 expression because GFP expression, which identifies cells over-expressing 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 induction of Tregs.

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

To assess the function of P4-iTregs in regulation of inflammatory diseases in vivo, we prepared myelin oligodendrocyte glycoprotein (MOG)-specific P4-iTregs (induced with IL2, TGFβ1 and P4) and control iTregs (induced with IL2 and TGFβ1) from naïve CD4⁺ T cells prepared from MOG-specific 2D2 transgenic mice, and compared their regulatory activities in vivo. As shown in Supplementary 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-iTregs were superior to control-iTregs in suppressing antigen-induced EAE (Fig. 6A and B). P4-iTregs were more efficient than control iTregs in decreasing infiltration of the spinal cord with inflammatory cells (Fig. 6C). Additionally, we examined if the P4-iTregs 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-iTregs were able to decrease the numbers of Th17 and Th1 cells in the CNS (Fig. 6D). Th17, but not Th1, frequencies were decreased in the CNS tissues (Supplementary Fig. 3B). P4-iTregs were statistically more efficient than control iTregs in suppression of the inflammatory cells in the brain.

We further investigated if the P4-iTregs 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-iTregs were more efficient than the control iTregs 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⁺ nTregs, P4-iTregs were somewhat more efficient in suppression of colitis and inflammatory T cells (Supplementary Fig. 4A and B). The frequencies of the two Treg populations were similar in various tissues (Supplementary Fig. 4C). Overall, the results demonstrate that P4 induces iTregs that are highly suppressive *in vivo*.

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

Tregs induced *in vitro* are unstable in expression of FoxP3 and readily revert back to non-Tregs [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 Tregs *in vitro* and *in vivo*, we examined if P4 stabilizes the FoxP3 expression in the T cell population. For this, we re-cultured the control iTregs and P4-iTregs in the presence or absence of P4 and assessed the expression of FoxP3 (Fig. 8A). We intentionally used a high concentration of TGF β 1 to prepare iTregs enriched with FoxP3⁺ T cells in this experiment. Upon re-culture, 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. The P4-iTregs were highly stable also *in vivo* in inflammatory conditions. Compared to control iTregs, almost all of which reverted back to FoxP3⁻ cells, a significant portion of P4-iTregs in the spleen and the draining lymph node remained as FoxP3⁺ T cells even 4 weeks after the cell transfer in myelin oligodendrocyte glycoprotein-induced experimental allergic encephalomyelitis mice (Fig. 8B) and T cell induced colitis mice in Rag1-deficient mice (Supplementary Fig. 5). These results indicate that P4 makes iTregs 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 Tregs but suppresses the generation of effector T helper cells [24, 25]. This function of P4 could be utilized to generate iTregs to control inflammatory diseases through generation of highly suppressive iTregs *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 iTregs is a major concern in utilizing these cells in clinical applications. The questions that we had regarding the induction of iTregs by P4 included: 1) Can the P4-induced iTregs be utilized to suppress inflammatory diseases *in vivo*? 2) Are classical P4 nuclear receptors involved in P4-mediated generation of iTregs? 3) What is the intracellular signaling process affected by P4 in T cells? Our results provided insights into these issues.

Our results indicate that iTregs induced by P4 are highly effective in suppressing inflammatory diseases. We used an EAE model and an IBD model. In both models, the iTregs induced by P4 were significantly better than control iTregs induced by TGF β 1 alone. iTregs appear to have variable activities in suppression of tissue inflammation [17–20]. Our results showed that iTregs have significant suppressive effects on EAE development. Although significant, the level of suppression was not high, and very few Tregs remained positive for FoxP3 in the EAE mice injected with iTregs. In contrast, P4-induced iTregs were effective in suppressing EAE and many more P4-iTregs than control iTregs remained positive for FoxP3. Control iTregs also had a suppressive activity on development of colitis at a moderate level. Compared to the control iTregs, P4-iTregs were more efficient in suppression of IBD. The superior in vivo suppressive functions are associated with two features of P4-induced iTregs. P4-induced iTregs are more stable and better sustain as FoxP3⁺ T cells than control iTregs. Another feature that can help them function better as Tregs in vivo would be high expression of LAP-TGF β 1, which is the major suppressive cytokine produced by Tregs [40]. We, however, did not demonstrate that this LAP-TGF β 1 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 iTregs at the expense of potentially inflammatory Th17 cells. This selective promotion of Tregs 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 iTregs 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 β 1 plus IL2 than wild type 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 Tregs. 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 μ g/g of placenta tissue. Induction of Tregs was detectable at 0.5 μ g/ml (1.6 μ M) of P4 and was maximal at 2 μ g/ml (6.4 μ M) in our study. Thus, the concentration (2 μ g/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 iTregs for therapeutic purposes, and we don't 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 Tregs are similar [42]. One apparent difference between the two species was that human adult peripheral blood naïve T cells were not amenable to make FoxP3⁺ T cells with P4 unlike their cord blood counterparts, whereas adult naïve T cells 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. Thus, direct comparison of the mouse and human data is difficult in this regard.

Effect of P4 on gene expression is complex and involves direct DNA binding and non-binding functions of PR [43]. 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 [44, 45]. The major function of mTORC1 is to activate p70 S6 kinase and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) for translation regulation [44, 45]. 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 Tregs [35, 36]. Our data indicate that P4 suppresses the mTORC1 signaling, and this is associated with enhanced generation of iTregs 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 iTregs 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 iTregs 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 [46]. 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. DO11.10 Rag2 (−/−) mice were purchased from Taconic Farms. 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 (Supplementary 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). 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. For preparation of nTregs (~70% were FoxP3⁺ cells), CD4⁺CD25⁺ cells were positively selected using an anti-mouse CD25 antibody (PC61) among the total CD4⁺ T cells.

Indicated tissues were harvested from 2 to 4 month-old female wild type or PR(−/−) mice. The tissues were digested with RPMI medium containing collagenase type 3 (2 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ) 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). For intracellular staining, cells stained with antibodies to CD4 and CD44 were activated for 4h with PMA, ionomycin and monensin for intracellular staining with antibodies to IL17 (TC11-18H10.1) and IFN γ (XMG1.2). P4 (4-Pregnene-3,20-dione, catalog # P8783, Sigma-Aldrich) 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 wild type, CD45.1 congenic, PR(−/−) mice, DO11.10 Rag2(−/−) mice, or 2D2 transgenic mice, were cultured for 6–7 days with concanavalin A (2.5 μ g/ml), OVA_{323–339} peptide (1 μ g/ml), myelin oligodendrocyte glycoprotein (MOG) antigen peptide (MOG_{35–55}; 1 μ g/ml) or anti-CD3/CD28 antibodies (plate-bound anti-CD3 at 1 μ g/ml, clone 145-2C11; soluble anti-CD28 at final 0.5 μ g/ml,

clone 37.51) in the presence or absence of P4 in RPMI medium. IL2 (100 U/ml) and TGF β 1 were added to induce Tregs. For activation with MOG_{35–55}, irradiated mouse splenocytes were used at 3 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-IL4, IL6 and TGF β 1) and cultured for 6–7 days as described previously [47]. Expression of FoxP3, LAP-TGF β 1 (TW7-16B4) or IL17/IFN γ was determined by a Canto II flow cytometer (BD Biosciences).

Methylation status of TSDR

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

Measurement of the in vitro suppressive activity

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4⁺CD25[−] T cells (target cells, 3×10⁴ cells/well) and iTregs as suppressors were co-cultured in round-bottom 96 well plates for 3 days at indicated ratios with anti-CD3 antibody (2 μ g/ml) and irradiated splenic APCs (9×10⁴ cells/well). iTregs were prepared by activation of naïve T cells with concanavalin A (2.5 μ g/ml) and IL2 (100 U/ml) for 5 days. P4 (2 μ g/ml or 6.4 μ M) and/or TGF β 1 (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 experimental allergic encephalomyelitis (EAE) model, 5×10⁶ P4-induced or control iTregs were injected i.v. into CD45.1⁺ congenic C57BL/6 mice and the mice were immunized with MOG_{35–55} peptide (100 μ g/mouse) in complete Freund's adjuvant. On the same day, pertussis toxin (100 ng/mouse) was injected i.v. The immunization was repeated 7 days later. The control iTregs (containing iFoxP3⁺ T cells) were prepared ex vivo by culturing naïve CD4⁺ T cells isolated from lymph node cells and splenocytes of 2D2 mice (Fig. 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 μ g/ml), TGF β 1 (0.5 ng/ml), and IL2 (25 U/ml). Irradiated splenocytes were added to activate the T cells for 6 days. P4 (2 μ g/ml or 6.4 μ M) was used to make P4-induced Tregs. 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 (3×10⁵/mouse) were injected i.p. together with CD45.2⁺ iTregs or nTregs (6×10⁵/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 iTregs versus naïve T cells. A hematoxylin and eosin (H&E) staining was performed on 6 μ m paraffin tissue sections to assess histological changes.

Assessment of in vitro stability of iTregs induced by P4

Naïve T cells were activated with concanavalin A (2.5 μ g/ml), IL2 (100 U/ml) and TGF β 1 (2 ng/ml) for 5–6 days in the presence of P4. The iTregs were re-cultured with concanavalin A

(2.5 µg/ml) or anti-CD3/CD28 beads (Miltenyi Biotec) and IL2 (100 U/ml) in the presence or absence of TGFβ1, 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) and IL2 (200 U/ml) in the presence of P4 for 2 hours. 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. 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) and anti-mouse CD4 antibody for 30 min at room temperature before flow cytometric analysis

Retroviral over-expression of Rheb

Murine Rheb was cloned into a LZRS-pBMN-IRES-GFP vector as described previously [48]. 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 IL2 (100 U/ml). Activated cells were infected with virus-containing conditioned medium in the presence of polybrene (6 µg/ml) by centrifugation at 1200g 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), a mixed Poisson model (time course EAE data), and a mixed linear regression model (time course IBD data) were used with STATA/IC version 11.1 (STATA, College Station, TX) to determine significance of the differences between groups. *p* values < or = 0.05 were considered significant. All error bars shown in this paper are SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Tregs	regulatory T cells
P4-iTregs	progesterone-induced Tregs
P4	progesterone
mTOR	mammalian target of rapamycin

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Fig. 1. Enhanced induction of FoxP3⁺ iTregs in the presence of P4

(A) Treg-depleted naïve CD4⁺ T cells were cultured for 6–7 days in the presence or absence of P4 (2 μg/ml) and TGFβ1. Concanavalin A (2.5 μg/ml) and IL2 (100 U/ml) were added. (B) Induction of P4-iTregs by activation with anti-CD3/28 antibodies or antigen peptide (OVA_{323–339}). Wild type C57BL/6 Treg-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. (C) Dose-dependent induction of FoxP3⁺ T cells in response to P4. The cells were cultured in the presence of TGFβ1 and IL2 and activated with anti-CD3/28 antibodies for 6 days. (D) Induction of LAP-TGFβ1 by P4 on T cells. TGFβ1 was used at 0.3 ng/ml for panel B and D. Representative or combined data (n=3–8/group) obtained from at least 3 independent experiments are shown. *Significant differences between control and P4 groups.

Fig. 2. P4 induces iTregs with potent suppressive activity but does not induce demethylation in the Treg-specific demethylated region (TSDR) in the FoxP3 gene

(A) P4-induced iTregs 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 Tregs was assessed based on dilution of CFSE fluorescence. (B) Methylation of the CpG sites in the TSDR region of nTregs, control Tregs and P4-Tregs. The iTregs were generated by culturing Treg-depleted naïve CD4⁺ T cells for 6 days in the presence or absence of P4 (2 µg/ml or 6.4 µM) and TGFβ1. Concanavalin A (2.5 µg/ml) and IL2 (100 U/ml) were used for T cell activation. Representative data obtained from 3 (panel A) and 2 (panel B) independent experiments are shown.

Fig. 3. P4-induced FoxP3 expression is partially dependent on nuclear progesterone receptor
 (A) Responses of PR (-/-) T cells to P4 in differentiation into iTregs. The culture condition was the same as for Fig. 1D. (B) Frequencies of FoxP3⁺ T cells in the uterus and other organs 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. Mean fluorescence intensity indicates FoxP3 expression levels in the T cells of wild type and PR (-/-) mice. The data were obtained from 4 independent experiments. *Significant differences between control and P4 groups. **Significant differences between wild type and PR (-/-) mice.

Fig. 4. P4 suppresses Th17 induction

(A) P4 effects on Th17 cell induction. Treg-depleted naïve CD4⁺ T cells, isolated from lymph nodes and spleen, were cultured for 6–7 days in a Th17 cell condition (anti-IFN γ , anti-IL4, IL6, TGF β 1, and concanavalin A) in the presence or absence of P4 (2 μ g/ml or 6.4 μ M). (B) PR is not required for P4-mediated suppression of Th17 cells. Naïve CD4⁺ T cells isolated from WT and PR(-/-) mice were cultured as in panel A and examined for induction of Th17 cells. Representative and combined data from 4–8 independent experiments are shown. *Significant differences between control and P4 groups.

Fig. 5. Activation of the mTOR pathway is suppressed by P4

(A) Rapamycin (3 nM) or P4 (2 μ g/ml or 6.4 μ M) was examined for their effects on phosphorylation of ribosomal S6 protein (Ser235/236) in antigen-primed T cells. Treg-depleted naïve CD4⁺ T cells, isolated from lymph nodes and spleen, were activated for 2 hours with anti-CD3/28 beads and IL2 (200 U/ml) in the presence of rapamycin or P4. Staining with an isotype control antibody is shown in gray. Representative data obtained from at least 3 independent experiments are shown. (B) The S6 protein phosphorylation in PR (-/-) T cells is not suppressed well by P4. The data in the graph are shown as % of control groups. (C) Rheb over-expression decreased the function of P4 in induction of LAP-TGFβ1⁺ Tregs. Rheb was over-expressed in Treg-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-TGFβ1⁺ Tregs was examined. Representative and combined data from 3 independent experiments are shown. Significant differences between indicated groups, * control and P4 groups, * or WT and PR(-/-) groups. **

Fig. 6. P4-induced iTregs are more effective than control iTregs in suppressing EAE development

(A) In vivo regulatory function of P4-induced antigen (MOG)-specific Tregs 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. (C) H&E histology of the spinal cords of the EAE mice treated with no Tregs, control (TGF β 1), or P4-iTregs. A representative image (n=17–21/group) from normal unimmunized mice without inflammation is also shown as a negative control. (D) P4-iTregs 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. The iTregs were prepared with a MOG peptide and irradiated splenocytes

from 2D2 mice (A) or MOG-immunized mice (D) as described in the materials and methods section. Data from two independent experiments were pooled. Significant differences between indicated groups,* from control mouse groups,* or from control iTreg groups.**

Fig. 7. P4-induced iTregs are more effective than control iTregs in suppressing murine colitis
 (A) P4-iTregs were effective in preventing inflammatory bowel disease in Rag1-deficient mice. iTregs 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 (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 Tregs (n=6–8). (C) Representative hematoxylin and eosin staining of the distal colon of the Rag1-deficient mice. *Significant differences between groups.

Fig. 8. P4-induced iTregs are more stable than control iTregs in FoxP3 expression

(A) Treg stability in vitro. Treg-depleted naïve CD4⁺ T cells were cultured for 6–7 days with concanavalin A in the presence of TGFβ1 (2 ng/ml) for control-iTregs or with TGFβ1 and P4 (2 μg/ml or 6.4 μM) for P4-iTregs. The T cells were re-cultured in the absence or presence of P4 and examined for FoxP3 expression. Representative data obtained from 5 independent experiments are shown. (B) Treg stability in vivo in an inflammatory condition. Frequencies of iTregs 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 combined data (n=9–10; pooled from 2 independent experiments) in a graph form are shown.