Progesterone Promotes Differentiation of Human Cord Blood Fetal T Cells into T Regulatory Cells but Suppresses Their Differentiation into Th17 Cells

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Progesterone Promotes Differentiation of Human Cord Blood Fetal T Cells into T Regulatory Cells but Suppresses Their Differentiation into Th17 Cells

Jee H. Lee, Benjamin Ulrich, Jungyoon Cho, Jeongho Park, and Chang H. Kim

Progesterone, a key female sex hormone with pleiotropic functions in maintenance of pregnancy, has profound effects on regulation of immune responses. We report in this work a novel function of progesterone in regulation of naive cord blood (CB) fetal T cell differentiation into key T regulatory cell (Treg) subsets. Progesterone drives allogeneic activation-induced differentiation of CB naive, but not adult peripheral blood, T cells into immune-suppressive Tregs, many of which express FoxP3. Compared with those induced in the absence of progesterone, the FoxP3+ T cells induced in the presence of progesterone highly expressed memory T cell markers. In this regard, the Treg compartment in progesterone-rich CB is enriched with memory-type FoxP3+ T cells. Moreover, CB APCs were more efficient than their peripheral blood counterparts in inducing FoxP3+ T cells. Another related function of progesterone that we discovered was to suppress the differentiation of CB CD4+ T cells into inflammation-associated Th17 cells. Progesterone enhanced activation of STAT5 in response to IL-2, whereas it decreased STAT3 activation in response to IL-6, which is in line with the selective activity of progesterone in generation of Tregs versus Th17 cells. Additionally, progesterone has a suppressive function on the expression of the IL-6 receptor by T cells. The results identified a novel role of progesterone in regulation of fetal T cell differentiation for promotion of immune tolerance.

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**Correspondence:** The online version of this article contains supplemental material.

**Abbreviations used in this article:** CB, cord blood; hIL-2, human IL-2; hIL-6, human IL-6; iFoxP3+, induced FoxP3+; iTreg, induced Treg; mPR, membrane progestin receptor; P4, progesterone; PB, peripheral blood; PR, progesterone receptor; Treg, T regulatory cell.

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T cell differentiation. Potential effects of this regulatory pathway on establishment of immune tolerance are discussed.

Materials and Methods
Preparation of cells
Mononuclear cells were prepared by density gradient centrifuge on Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) from human CB and adult PB as described previously (33). CD4+ T cells were isolated by the CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA) and further processed for depletion of CD25+ T cells. The isolated CB CD4+CD25− naive T cells routinely had a purity >98%. For isolation of PB CD4+CD45RA+CD45RO− naive T cells, total CD4+ T cells were further depleted with CD45RO+, CD69+, and CD25+ T cells as described previously (34). For allogeneic APCs, CD3−CD56− CB or adult PB mononuclear cells were used after irradiation (2000 rad). The use of human CB and PB for this study was approved by the Institutional Review Board at Purdue University.

In vitro T cell differentiation in response to P4
Neonatal CB and adult PB naive CD4+ T cells were activated with one of the T cell activators including anti-CD3– and anti-CD28–coated beads (5 μl/million cells; Miltenyi Biotec), PHA (5 μg/ml), or irradiated CD3−CD56− allogeneic PB or CB mononuclear cells as APCs for 6–7 d. IL-2 (25 U/ml) was added for generation of Tregs. All experiments were performed with phenol red-free RPMI 1640 medium supplemented with charcoal/dextran-treated FBS (10%; HyClone). Depending on the activity of each batch, TGF-β1 was used in the concentration range from 25 to 500

FIGURE 1. P4 drives the differentiation of CB CD4+ T cells into highly suppressive Tregs. A, Treg-depleted CB fetal (CD4+CD25−) T cells were activated with beads coated with Abs to CD3 and CD28, P4 (2 μg/ml), and IL-2 for 7 d, and frequencies of FoxP3+ T cells were determined by flow cytometry. TGF-β1 was added as indicated. B, Microscopic detection of FoxP3 expression in CB T cells stimulated in the presence of P4 and/or TGF-β1 (original magnification ×20). C, A dose-dependent response of T cells to P4 in FoxP3 induction in the presence of TGF-β1. D, The suppressive function of control and P4-treated CB T cells on the proliferation of CFSE-labeled CB CD4+CD25− naive T cells was examined. To prepare suppressors, CB naive (CD4+CD25−) T cells were cultured in the presence of IL-2 (control) or IL-2 and P4 (P4) for 6–7 d. TGF-β1 was added as indicated. The suppressors were cultured with CFSE-labeled target T cells for 5 d, and the CFSE intensity of target cells was determined by flow cytometry. E and F, RU486, a PR antagonist, was effective in blocking the expression of FoxP3 protein (E) and mRNA (F) induced by P4. Combined data are shown in the graphs in A (n = 9), C (n = 10), E (n = 7), and F (n = 3). *Significant differences between the two groups with p values or from the TGF-β1–alone control group.
FIGURE 2. CB, but not adult PB, naive T cells differentiate into FoxP3+ Tregs in response to P4. A, CB naive T cells and adult PB naive CD4+ T cells were compared for their response to P4. T cells were activated with beads coated with Abs to CD3 and CD28, P4 (2 μg/ml), and IL-2 for 7 d. TGF-β1 was added to all cultures. After culture, frequencies of FoxP3+ T cells among CD4+ T cells were determined by flow cytometry (n = 8–10). B, P4 promotes allogeneic Ag priming-induced differentiation of CB T cells into FoxP3+ T cells. Naive CD4+ T cells isolated from the indicated sources were cocultured with allogeneic APCs (irradiated mononuclear cells) obtained from CB or PB for 7 d in the presence of indicated concentrations of P4 (n = 4–6). Significant differences from the *control point (0 μg/ml) or from **PB.

pg/ml to induce FoxP3 at a moderate level (~30%). The cultured T cells were examined for expression of the FoxP3 protein by intracellular staining with anti-FoxP3 Ab (clone 236A/E7 for human; eBioscience, San Diego, CA) and of FoxP3 mRNA by real-time PCR with the primers listed in Supplemental Table I.

For induction of Th17 cells, CB naive CD4+ T cells were activated with anti-CD3/CD28 beads (Miltenyi Biotec) for 6–7 d in RPMI 1640 medium supplemented with FBS (10%), IL-23 (25 ng/ml; R&D Systems), IL-1β (10 ng/ml; PeproTech, Rocky Hill, NJ), TGF-β1 (1 ng/ml; PeproTech), anti-IL-4 (10 μg/ml; MP4-25D2; BioLegend), and anti–IFN-γ (10 μg/ml; MD-1; BioLegend). P4 was added at 2 μg/ml from the beginning of the culture. T cells were stained with anti-CD4 (RPA-T4) and then activated with PMA (50 ng/ml) and ionomycin (1 μM) for 4 h in the presence of monensin (2 μg/ml) before staining with Abs to IL-17 and IFN-γ as previously described (35).

Flow cytometry to determine surface Ag phenotype and STAT phosphorylation
Fresh or cultured T cells were examined for expression of CD4 (RPA-4), FoxP3 (PCH101), CD45RA (H1001), CD45R0 (UCHL1), CD49f (GoH3), CD58 (IC5), CD45RB (MT4), CD25 (BC96), CD62L (DREG56), and FoxP3 (PCH101), CD45RA (HI100), CD45RO (UCHL1), CD49f (GoH3), FoxP3 was determined by Canto II.

Assessment of suppressive activity of the P4-induced FoxP3+ T cells
CB CD4+CD25+ T cells (responders, 5 × 10^5 cells/well) and indicated cultured T cells as suppressors were cocultured in 96-well plates for 5 d at indicated ratios in the presence of anti-CD3/CD28 beads. The suppressors were prepared by a culture with IL-2 (25 U/ml) for 6–7 d. Additionally, TGF-β1 (1 ng/ml) and/or P4 (2 μg/ml) were added as indicated. CB CD4+CD25+ T cells were labeled with CFSE and cocultured with indicated numbers of suppressors. Dilution of CFSE was determined by flow cytometry.

Impact of P4 on T cell differentiation into iTregs in the presence of IL-6
Human CB naive cells were activated with anti-CD3/CD28 beads together with or without P4 (2 μg/ml) in RPMI 1640 medium containing charcoal/dextran-treated FBS (10%), IL-6 (25 U/ml), and TGF-β1 for 6–7 d. IL-6 and/or anti–IL-6R neutralizing Ab (MAB227, 10 μg/ml) were added to the culture. The cultured cells were harvested and stained with anti-human CD4, anti-human CD25, and anti-human FoxP3 Abs. The expression of FoxP3 was determined by Canto II.

Immunofluorescence staining and confocal microscopy
The cultured T cells were stained with Abs for surface CD4 (clone RPA-T4) and intracellular FoxP3 (clone 236A/E7). The cells were cytopsin on slide glasses, and the expression of surface CD4 and nuclear FoxP3 was documented by confocal microscopy (Zeiss LSM 710).

Microarray gene expression analysis
Human CB and PB naive T cells were cultured for 5 d with anti-CD3/CD28 beads (Miltenyi Biotec) in the presence or absence of P4 (2 μg/ml) and/or

FIGURE 3. Impact of P4 on stability of FoxP3 expression in T cells. CB naive CD4+CD25− T cells were activated with beads coated with Abs to CD3 and CD28, P4 (2 μg/ml), and IL-2 for 3 or 6 d and then recultured with P4 and/or TGF-β1 for 6 d. Anti-CD3/CD28 and IL-2 were used to activate the T cells. FoxP3 expression was measured after the second culture. Combined data from three to four experiments are shown. *Significant differences from the respective control groups (no P4).
TGF-β1 (150 pg/ml) in the RPMI 1640 medium supplemented with charcoal/dextran-treated FBS (10%) and IL-2 (25 U/ml). The cultured cells were harvested, and the total RNA was isolated using RNeasy kit (Qiagen). Total RNA, isolated from the cultured CD4⁺ T cells, was hybridized to Human Genome U133 Plus 2.0 Array chips (Affymetrix) by Purdue Genomics Laboratory. These arrays contain >54,000 cDNA spots corresponding with human sequence-verified transcripts. Raw intensity values were obtained with the GCOS software (Affymetrix) and normalized with the expression values of a housekeeping gene (GAPDH). Selection and filtering of high-quality genes were based on a 2-fold or greater differential in expression up or down between two conditions of comparison. Further selection was based on reproducibility between technical replicates, and transcripts without consistent results or with signal intensity <50 were dismissed. The gene expression values were visualized with the multiplot module of the GenePattern genomic analysis platform (http://www.broad.mit.edu/cancer/software/genepattern) and the TreeView module from EisenSoftware (http://rana.lbl.gov/EisenSoftware.htm). The raw and processed array data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE22025.

**Real-time PCR analysis of mRNA expression**

cDNA was made and real-time quantitative PCR was performed on a 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green Master Mix (Applied Biosystems) as described previously (36). Normalized expression was calculated based on threshold cycle values with β-actin used as an internal control. The primers used for real-time PCR are shown in Supplemental Table I.

**Statistical analysis**

All experiments were performed multiple times to confirm their reproducibility. For combined data from multiple experiments, Student paired

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**FIGURE 4.** The CB Treg compartment is enriched with memory-type FoxP3⁺ T cells. A, Frequencies of naive versus memory FoxP3⁺ and FoxP3⁻ T cells in fresh CB. B, Comparison of CB and adult PB in frequencies of naive and memory FoxP3⁺ T cells. C, Surface phenotype of CB FoxP3⁺ T cells. Combined data (n = 4–8) are shown in a graph form. *Significant differences between paired groups or from FoxP3⁻ T cells.
two-tailed $t$ test was used, and $p$ values $\leq 0.05$ were considered significant. For combined data, the error bars are SEM. For the data that are difficult to combine, representative data are shown with error bars showing differences between duplicated measurements.

**Results**

*P4 promotes the differentiation of human CB naive T cells into Tregs with a highly regulatory function*

To determine if P4 enhances FoxP3$^+$ Treg differentiation, CB naive T cells were activated in the presence or absence of P4. We supplemented the culture with TGF-$\beta$1 at a minimally active concentration, which is known to induce iFoxP3$^+$ T cells. P4 had a positive effect on generation of iFoxP3$^+$ T cells (Fig. 1A). The FoxP3 induction in response to P4 occurred also in the absence of exogenous TGF-$\beta$1 in some experiments, but it was more reliable and efficient in the presence of TGF-$\beta$1. The FoxP3 protein expressed in response to P4 was localized to the nuclei of T cells as expected (Fig. 1B). The effect of P4 was a dose-dependent one, peaking at around 2 $\mu$g/ml P4 (Fig. 1C).

It has been reported that induced human FoxP3$^+$ T cells with TGF-$\beta$1 lack the suppressive activity characteristic for Tregs (37). Therefore, simple induction of FoxP3 appears not sufficient to make human T cells into Tregs, which is better defined functionally. Because of this reason, we investigated if the P4-induced FoxP3$^+$ T cells have a real Treg-like suppressive function. We determined the ability of P4-induced Tregs to suppress the proliferation of target T cells (CFSE-stained CB CD4$^+$CD25$^-$ T cells). Compared with IL-2–treated T cells, P4 plus IL-2–treated T cells displayed a greater suppressing activity on the proliferation of target (CD4$^+$CD25$^-$) T cells compared with that of T cells treated with TGF-$\beta$1 alone (Fig. 1D). RU486, an antagonist for several different

![Figure 5](http://www.jimmunol.org/)
PRs, fully suppressed the P4-induced expression of the FoxP3 protein and transcription of the FoxP3 gene (Fig. 1E, 1F).

**Adult PB naive T cells are resistant to P4-induced conversion into FoxP3+ T cells**

It has previously been reported that CB is a better source of Treg progenitors compared with PB (38). We examined if CB T cells are different from PB T cells in response to P4. Compared with the highly sensitive CB T cells, adult PB naive T cells were largely unresponsive to P4 (Fig. 2A).

To determine if P4 can selectively promote the induction of FoxP3+ T cells from CB versus PB naive T cells during allogeneic responses between T cells and APCs, we cocultured naive CD4+ T cells and irradiated T cell-depleted allogeneic CB or PB mononuclear cells isolated from different individuals (Fig. 2B). P4 significantly increased the induction of FoxP3+ T cells during the allogeneic priming of CB T cells. In contrast, adult PB naive T cells were again poorly responsive. CB APCs were more potent than adult APCs in induction of P4-induced FoxP3+ T cells (Fig. 2B).

Next, we asked if CB naive T cells would remain responsive to P4 even after previous Ag priming events. To address this, CB T cells were cultured first with or without P4 and then recultured with P4 and/or TGF-β1 (Fig. 3). P4 was unable to induce FoxP3 expression once the CB naive T cells were preactivated. Initial exposure to P4 was critical to induce FoxP3+ T cells at high levels by P4 upon subsequent activation with TGF-β1 alone or P4 and TGF-β1. Three days of initial P4 exposure was sufficient to induce this effect. The results suggest that CB T cells indeed lose the ability to respond to P4 and TGF-β1 if they are previously activated in the absence of P4.

**P4-induced iFoxP3+ T cells are related to the memory-type natural CB FoxP3+ T cells**

Human CB CD4+ T cells, although known to be mostly composed of naive cells, contain a significant memory (CD45RA-CD45RO+) cell population, suggesting a history of ongoing Ag-priming events in fetuses (Fig. 4A). This can be induced preferentially by allogeneic immune responses between fetal T cells and maternal APCs or fetal APCs presenting maternal Ags. Notably, 30–40% of these memory T cells were FoxP3+ T cells (Fig. 4A, 4B). This is considered very high compared with the low frequency (~10%) of FoxP3+ T cells among adult PB memory T cells (Fig. 4B). Whereas ~30% of FoxP3+ T cells were CD45RA-CD45RO+ memory T cells, only 1–5% of FoxP3+ T cells were CD45RA-CD45RO+ (Fig. 4A). These results suggest that many CB FoxP3+ T cells may have been Ag-primed and assumed the memory T cell phenotype. These memory-type CB FoxP3+ T cells expressed CD49f (integrin α6) and CD58 (LFA-3), the Ags typically associated with Ag-primed T cells (Fig. 4C). Otherwise, these FoxP3+ T cells were CD25+CTLA4+, a phenotype typically associated with the FoxP3+ T cell population.

Most P4-induced iFoxP3+ T cells, but not control iFoxP3+ T cells generated in vitro, were CD45RO-CD45RA- cells (Fig. 5A), and this phenotype is similar to the memory-type FoxP3+ T cells naturally present in CB (Fig. 4). In contrast, the T cells Ag-primed without exogenous P4 (but in the presence of TGF-β1) were mostly T cells of intermediary CD45RO-CD45RA2 phenotype, suggesting that P4 has a profound regulatory effect on expression of the naive and memory cell-associated Ags as well. Moreover, P4-induced FoxP3+ T cells were higher than control iFoxP3+ T cells in expression of CTLA4 and CD69, but lower in expression of...
CD45RB and CD62L, a feature consistent with recently activated memory FoxP3+ T cells (Fig. 5B). Expression of another Treg-associated molecule, 4-1BB, was somewhat decreased on these cells compared with that on control iFoxP3+ T cells.

**P4 suppresses the generation of Th17 cells**

Th17 cells and FoxP3+ T cells, although very different in their functions, are related in their differentiation. Both of the populations are induced by TGF-β1. However, they are different in that IL-2 promotes FoxP3+ T cells and suppresses Th17 cell differentiation, whereas inflammatory cytokines such as IL-6 that induce Th17 cells suppress FoxP3+ T cell development. We therefore examined if P4 has any role in differentiation of CB T cells into Th17 cells in response to inflammatory cytokines. For this, we cultured naive CB CD4+ T cells in a Th17 cell induction condition in the presence and absence of P4. P4 dramatically reduced the conversion of naive T cells into Th17 cells (Fig. 6). The effect of P4 is similar to that of retinoic acid, which is known to suppress Th17 cells. Along with IL-17A, expression of Th17 cell-associated IL-17F, IL-21, and RORC transcripts was decreased by P4 (Fig. 6B). Although not statistically significant, other genes such as IL-23R, STAT3, AHR, and CCR6 were decreased, too. Expression of IL-22, however, was not suppressed by P4.

**Potential mechanisms for P4 regulation of T cell differentiation through modulation of STAT5, STAT3, and IL-6R**

STAT5 A/B proteins are key molecules that regulate T cell differentiation into FoxP3+ T cells and Th17 cells. Activation of STAT5 by IL-2 promotes the generation of FoxP3+ T cells at the expense of Th17 cells. We found that P4 increases the phosphorylation of STAT5 A/B in the CB T cells induced by IL-2 (Fig. 7A). P4 by itself, however, did not induce phosphorylation of STAT5 A/B. We examined also the activation of STAT3 because this molecule is central to transmission of signals to induce Th17 cells and suppress Tregs. Upon stimulation with IL-6, T cells cultured with P4 had decreased phosphorylation of STAT3 compared with that of the T cells cultured in the absence of P4 (Fig. 7B). Thus, these results show that STAT3 and STAT5 are downstream targets of P4 for regulation of FoxP3+ T cells and Th17 cells.

To gain more insights into the P4 regulation of T cell differentiation, we examined the global gene expression pattern of CB T cells regulated by P4. We found 325–347 genes upregulated or downregulated by P4 either in the presence or absence of exogenous TGF-β1 (Fig. 8A, Supplemental Fig. 1A, B). PB T cells were relatively unresponsive with only 30–70 genes regulated by P4. IL-6R expression was greatly downregulated by P4 in CB, but not PB, T cells (Fig. 8A). IL-6R mediates the IL-6 signal, which suppresses the induction of FoxP3+ T cells but enhances the induction of Th17 cells (39, 40). We confirmed that IL-6R mRNA and protein are decreased by P4 (Fig. 8B, 8C). Consistently, iFoxP3+ T cells were made even in the presence of IL-6 when P4 was added to the culture (Fig. 8D). Also, the decrease in IL-6R expression by P4 accounts for the decreased induction of Th17 cells in response to IL-6 (Fig. 8E).

The following genes regulated by P4 have been verified by real-time PCR (Supplemental Fig. 1C): growth factor independent 1 transcription repressor (GFI1, a suppressor of Th17 cell induction) (41), ankyrin repeat and SOCS box-containing 2 (ASB2, a regulator for degradation of SOCS proteins) (42), CD38 (a cyclic ADP ribose hydrolase), EBI3 (the common subunit of IL-27 and IL-35), TNFSF13B (also called BAFF or BLyS), leucine zipper

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**FIGURE 7.** P4 enhances STAT5 activation in response to IL-2, whereas it suppresses STAT3 activation in response to IL-6. CB CD4+CD25+ T cells were activated with anti-CD3/CD28 beads in the presence or absence of P4 for 20 h. The activated cells were stimulated with recombinant hIL-2 (200 U/ml) or hIL-6 (50 ng/ml) for 30 min and stained with an (A) anti-STAT5 (pY694) or (B) anti-STAT3 (pY705) Ab, respectively. A control Ab was used to identify the basal staining levels for the T cells, which are shown as filled gray histograms. Representative and combined data (n = 3) are shown. *Significant differences between the groups.
transcription factor-like 1 (LZTFL1) (43), methyltransferase like 7A (METTL7A), hypermethylated in cancer 1 protein (HIC1), growth factor independent 1 transcription repressor (GFI1) (44), SOCS1, SOCS2, SOCS3, CCL5, 29,59-oligoadenylate synthetase 1 (OAS1), and IFN-induced protein with tetratricopeptide repeats 3 (IFIT3). Among these, SOCS1 and SOCS2 were downregulated, whereas others were upregulated. A more extensive list can be found in Supplemental Fig. 1B.

**Discussion**

In this study, we investigated the P4 effect on CB fetal T cell differentiation into immune-suppressive Tregs and inflammatory Th17 cells. Through the study, we gained novel information regarding the function of P4 in regulation of T cell differentiation. First, we found that P4 has a positive effect on human naive T cell differentiation into Tregs expressing FoxP3. Notably, CB T cells were highly responsive to P4 in this process, whereas adult PB T cells were largely unresponsive to P4. Second, whereas P4 promotes the differentiation of naive T cells into FoxP3+ T cells, P4 has an inhibitory effect on CB T cell differentiation into Th17 cells. P4 acts on neonatal T cells undergoing Ag priming and changes their gene expression program to steer the T cell differentiation into tolerogenic Tregs but suppresses the generation of potentially inflammatory effector T cells.

Several studies have proposed the link between nuclear hormones and FoxP3+ T cells. The best-studied example to date is the role of retinoic acid in induction of FoxP3+ T cells (34, 45–47). Retinoic acid by itself is a weak inducer of FoxP3+ T cells but has an inhibitory effect on CB T cell differentiation into Th17 cells. P4 acts on neonatal T cells undergoing Ag priming and changes their gene expression program to steer the T cell differentiation into tolerogenic Tregs but suppresses the generation of potentially inflammatory effector T cells.
on generation of Th17 cells. Also, P4 is implicated in induction and expansion of CD4+CD25+ Tregs in the uteruses of pregnant mice (48). Others reported that Tregs were decreased in the circulation of pregnant females and proposed that P4 may negatively regulate T cell conversion into Tregs (49). This study, however, failed to determine the direct effect of P4 on Treg-depleted naive T cells and, thus, appears to be inconclusive. Also, the circulating Tregs may not faithfully represent the Tregs that are induced in response to P4 in specific tissues. Other nuclear hormones that are implicated in induction or expansion of Tregs are estrogen and vitamin D (50, 51). Therefore, nuclear hormone receptors as a group appear to play important roles in induction of immune tolerance.

Separate from the function of P4, we observed that APCs of CB provide signals conducive for generation of Tregs. When CB T cells are Ag-primed by allogeneic APCs, CB APCs are significantly more efficient than PB APCs in inducing FoxP3+ T cells. All of the characteristics of CB T cells and APCs would work together to make the immune responses induced by CB cells more tolerogenic and less inflammatory.

Our results have implications in a number of biological processes. One important process is regulation of immune responses and T cell differentiation during fetal development. The serum concentration of P4 at near-term pregnancy can reach ~150 ng/ml in maternal circulation, ~400 ng/ml in CB, and 300–3000 ng/ml in the human placental tissue (3, 52). Thus, up to ~3000 ng/ml P4 is considered within the physiological concentration range. Importantly, the doses of P4 that we used in this study are within this range. The P4-treated T cells were more suppressive than control iTregs induced with TGF-β1, and this suggests that P4 fundamentally changes the function of most T cells beyond induction of FoxP3 expression in some cells. P4 has the potential to suppress T cell responses in both pregnant females and fetuses. The difficulty of adult T cells to become Tregs in response to P4, however, suggests that the P4 function is more important in fetuses and newborns than in pregnant females. Therefore, a hypothetical role for P4-induced induction of Tregs and suppression of Th17 cells is to promote immune tolerance in the early life.

We identified increased STAT5 activation and decreased STAT3 activation and IL-6R expression by P4 as potential mechanisms for the P4-regulated differentiation of T cells. STAT5 activation in response to IL-2 promotes T cell differentiation into FoxP3+ T cells but restrains the process of differentiation into Th17 cells. IL-6R, of course, is essential to receive the IL-6 signal, which is, in contrast to IL-2 and STAT5, a major signal to shift T cell differentiation toward the generation of Th17 cells away from FoxP3+ T cells. In this regard, it has been reported that P4 induces the expression and activation of STAT5 in breast cells (53, 54). STAT3 is a central player in delivering the signals from IL-6 and IL-23 in generation of Th17 cells and suppression of Tregs. Therefore, dampened activation of STAT3 in conjunction with enhanced activation of STAT5 would make the cell signaling system conducive to generation of Tregs and suppress Th17 cell development. It may not be just coincidental that decreased IL-6R is linked to retinoic acid-mediated regulation of T cell differentiation in mice as well (55). It appears that there are common mechanisms for the action of nuclear hormone receptor ligands in regulation of T cell differentiation. In addition to STAT3/5 and IL-6R, additional genes and molecules are likely to be involved in the regulation of fetal T cells by P4. In this regard, we identified many genes that are regulated by P4 through a genome-wide microarray study.

The results of the genome-wide microarray study show the differential responses between CB and PB T cells in response to P4. CB T cells were highly responsive in upregulating or down-regulating genes, whereas PB T cells did not significantly change their gene expression program in response to P4. It would be interesting to study in the future how the genes in CB and PB T cells are differentially regulated at the molecular level. Among the genes upregulated with P4, TNFSF13B/BAFF is known to expand CD4+CD25Foxp3+ Tregs (56). EB13, a subunit of IL35 and induced by P4, is a key effector cytokine of Tregs to suppress target cells (57, 58). SOCS1, which negatively regulates Tregs (59), was decreased by P4. CD38, important for homeostasis of Tregs through regulation of NAD (60), was increased by P4. Some of the genes such as OAS1, RGS1, and TNFSF13B, highly induced in response to P4, were among the genes induced in Tregs derived from fetal stem and progenitor cells (25). The roles of the P4-regulated molecules in control of T cell differentiation and function remain to be determined.

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Disclosures
The authors have no financial conflicts of interest.

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9. Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner,
Supplemental File

Supplemental Table 1. Primers used in the study

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Supplemental Figures

**Fig. 1. Genes that are regulated by P4 in CB T cells.** CB and PB naïve T cells, cultured in the presence or absence of P4 and/or TGFβ1 for 5 days, were compared for global gene expression. Two sets of the experiments were compared for identification of genes that were reproducibly induced or suppressed. (A) Multi-plots with highlighted genes reproducible up or down-regulated in response to P4 in the presence or absence of TGFβ1. (B) Treeviews of gene groups up- or down-regulated by P4 in T cells. Group A and B display genes down-regulated by P4 primarily in PB and CB respectively. Group C has genes up-regulated by P4 in both. Group D1 and D2 display genes that are up-regulated primarily in CB. Two sets of data in five gene groups are shown. (C) A real time RT-PCR assay on CB T cells was performed for the selected genes. The data are representative of 2-3 independent experiments. Relative expression values after normalized with β-actin levels are shown. The error bars are differences of duplicated measurements.
Supplemental Figure 1
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