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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[1]

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Abstract

Progesterone, a key female sex hormone with pleiotropic functions in maintenance of pregnancy, has profound effects on regulation of immune responses. We report here a novel function of progesterone in regulation of naïve cord blood (CB) fetal T cell differentiation into key regulatory T cell subsets. Progesterone drives allogeneic activation-induced differentiation of CB naive, but not adult peripheral blood (PB), T cells into immune suppressive T regulatory cells (Tregs), many of which express FoxP3. Compared to 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 antigen presenting cells were more efficient in inducing FoxP3$^+$ T cells than their PB counterparts. 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 while 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.

Introduction

Progesterone (P4)2 is a multifunctional female sex hormone, promoting the development of mammary glands, ovulation, implantation of embryos, and maintenance of pregnancy (1). P4 is produced by granulosa cells and corpus luteum in the ovary. During pregnancy, the placenta becomes a major organ for P4 production. P4 can be produced also by cord blood (CB)2 erythroblasts and is present at high levels in CB (2, 3). While the mechanism is still unclear, P4 is implicated in dampening immune responses to fetal and maternal antigens (4). P4 is metabolized into 17-hydroxyprogesterone, which can be eventually converted into other nuclear hormones including glucocorticoids, testosterone, and estradiol (5). P4 or its metabolic derivatives work through a number of different receptors. These include nuclear progesterone receptors (PR-A, PR-B and PR-C), membrane progestin receptors (mPRα, mPRβ, and mPRγ), progesterone receptor membrane component-1 and 2 (PGRMC1 and

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[2]Abbreviations: CB, cord blood; PB, peripheral blood; P4, progesterone; PR, progesterone receptor; iFoxP3$^+$ cells, induced FoxP3$^+$ cells; Tregs, T regulatory cells; iTregs, induced Tregs.

PGRMC2), and glucocorticoid receptor (GR) (6–11). It has been documented that PRs and mPRs are expressed by T cells (10, 12, 13).

T regulatory cells (Tregs)2 are heterogeneous and are functionally defined by their suppressive function on non-Treg cells. Tregs can be classified into natural and induced (i) Tregs. Some Tregs co-express FoxP3 and CD25 and have been studied extensively. Tregs play important roles in suppression of inflammatory and autoimmune responses (14–17). $FoxP3^+$ Tregs are increased during pregnancy in both maternal and fetal systems in response to allogeneic antigens (18, 19). TGFβ1 and IL-2 play central roles in induction of induced $FoxP3^+$ ($iFoxP3^+$)2 Tregs (20–22). Fetal T cells have an increased propensity to differentiate into Tregs in a TGFβ1-dependent fashion (23, 24), and fetal T cell progenitor cells can more efficiently make Tregs compared to adult progenitor cells (25). Signal transducers and activators of transcription (STAT) 5A/B are involved in the IL-2-dependent induction of $FoxP3^+$ T cells but restrain the generation of Th17 cells (26, 27). Th17 cells are defined by their ability to produce IL-17 and are associated with immune responses to extracellular pathogens and tissue-specific autoimmune diseases (28, 29). Th17 cells are mostly induced in the periphery from naïve T cells upon antigen priming. It has been well established that inflammatory cytokines such as IL-6, IL-23, IL-1β and TNF-α promote the generation of Th17 cells during antigen priming (30). Transcription factors, such as STAT3, RORγt, RORα and aryl hydrocarbon receptor (AHR), are implicated in Th17 cell development (28, 30–32).

Because P4 is greatly increased in CB and fetal circulation and plays important roles in suppression of immune responses, we investigated the possibility that P4 is actively involved in generation of Tregs and Th17 cells from CB naïve T cells. Our study revealed novel functions of P4 in regulation of immune responses. P4 induces naïve CB T cell differentiation into immune-suppressive Tregs, but it suppresses their differentiation into inflammatory Th17 cells. Interestingly, the P4-mediated induction of Tregs efficiently occurs with CB naïve T cells but not with adult peripheral blood (PB)2 T cells. We provide potential mechanisms involving STAT3/5 proteins and IL-6R in selective regulation of CB T cell differentiation. Potential impacts 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 Inc. Auburn, CA) and further processed for depletion of $CD25^+$ T cells. The isolated CB $CD4^+CD25^-$ naïve T cells routinely had purity greater than 98%. For isolation of PB $CD4^+$ $CD45RA^+CD45RO^-$ naïve T cells, total $CD4^+$ T cells were further depleted with $CD45RO^+$, $CD69^+$, and $CD25^+$ T cells as described previously (34). For allogeneic antigen presenting cells, $CD3^-CD56^-$ CB or adult PB mononuclear cells were used after irradiation (2000 Rad). The use of human CB and PB for this study has been 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 & anti-CD28-coated beads (5 μl/million cells; Miltenyi Biotec Inc), phytohemagglutinin (PHA, 5 μg/ml), or irradiated $CD3^-CD56^-$ allogeneic PB or CB mononuclear cells as antigen presenting cells for 6–7 days. IL-2 (25 U/ml) was added for

generation of Tregs. All experiments were performed with phenol red-free RPMI medium supplemented with charcoal/dextran-treated fetal bovine serum (10%; HyClone). Depending on the activity of each batch, TGFβ1 was used in the concentration range from 25 to 500 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 antibody (clone 236A/E7 for human, eBioscience, San Diego, CA) and of FoxP3 mRNA by real time PCR with the primers listed in Supplemental Table 1.

For induction of Th17 cells, CB naive $CD4^+$ T cells were activated with anti-CD3/CD28 beads (Miltenyi Biotec Inc) for 6–7 days in RPMI medium supplemented with FBS (10%), IL-23 (25 ng/ml; R&D Systems), IL-1β (10 ng/ml; PeproTech, Inc. Rocky Hill, NJ), TGFβ1 (1 ng/ml; PeproTech), anti-IL-4 (10 μg/ml; MP4-25D2, BioLegend Inc), and anti-IFNγ (10 μg/ml; MD-1, BioLegend Inc). 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 antibodies to IL-17 and IFNγ as previously described (35).

Flow cytometry to determine surface antigen phenotype and STAT phosphorylation

Fresh or cultured T cells were examined for expression of CD4 (RPA-4), FoxP3 (PCH101), CD45RA (HI100), CD45RO (UCHL1), CD49f (GoH3), CD58 (1C3), CD45RB (MT4), CD25 (BC96), CD62L (DREG56), and CTLA4 (BNI3). A BD Canto II was used to determine the expression of the antigens. For detection of phosphorylated STAT5A/B or STAT3, human CB $CD4^+$ T cells were first activated for 20 hours with anti-CD3/CD28 beads (Miltenyi Biotec Inc) and then stimulated with hIL-2 (200 U/ml) or hIL-6 (50 ng/ml) for 30 min. The cells were then fixed in 4% paraformaldehyde for 30 min followed by permeabilization in BD Phosflow Perm buffer III. Permeabilized cells were stained with anti-STAT5A/B (pY694) or anti-STAT3 (pY705) antibody (BD Biosciences, clone 47 and 4/P-STAT3) along with an anti-CD4 antibody.

Assessment of suppressive activity of the P4-induced $FoxP3^+$ T cells

CB $CD4^+CD25^-$ T cells (responders, 5×10^4 cells/well) and indicated cultured T cells as suppressors were co-cultured in 96-well plates for 5 days 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 days. Additionally, TGFβ1 (1 ng/ml) and/or P4 (2 μg/ml) were added as indicated. CB $CD4^+CD25^-$ T cells were labeled with CFSE and co-cultured 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 naïve cells were activated with anti-CD3/CD28 beads together with or without P4 (2 μg/ml) in RPMI medium containing charcoal/dextran-treated FBS (10%), hIL-2 (25 U/ml) and TGFβ1 for 6–7 days. hIL-6 and/or anti-IL-6R neutralizing antibody (MAB227, 10 μg/ml) were added to the culture. The cultured cells were harvested and stained with anti-hCD4, anti-hCD25 and anti-hFoxP3 antibodies. The expression of FoxP3 was determined by Canto II.

Immunofluorescence staining and confocal microscopy

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

Microarray gene expression analysis

Human CB and PB naïve T cells were cultured for 5 days with anti-CD3/CD28 beads (Miltenyi Biotec) in the presence or absence of P4 (2 μg/ml) and/or TGFβ1 (150 pg/ml) in the RPMI medium supplemented with charcoal/dextran-treated FBS (10%) and hIL-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, was hybridized to Human Genome U133 Plus 2.0 Array chips (Affymetrix, Inc.) by Purdue Genomics Laboratory. These arrays contain over 54,000 cDNA spots corresponding to human sequence verified transcripts. Raw intensity values were obtained with the GCOS software (Affymetrix, Inc.) and normalized with the expression values of a housekeeping gene (GAPDH). Selection and filtering of high quality genes were based on a two-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 lower than 50 were dismissed. The gene expression values were visualized with the multiplot module of the GenePattern genomic analysis platform (www.broad.mit.edu/cancer/software/genepattern) and TreeView module from EisenSoftware (http://rana.lbl.gov/EisenSoftware.htm). The raw and processed array data have been deposited at the GEO database (http://www.ncbi.nlm.nih.gov/geo/) (submission numbers: GSE22014, GSE22015, and 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 Ct values with β-actin used as an internal control. The primers used for real time PCR are shown in Supplemental Table 1.

Statistical analysis

All experiments were performed multiple times to confirm their reproducibility. For combined data from multiple experiments, student's paired 2-tailed *t* test was used. *p* values < or = 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 cord blood 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 TGFβ1 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 TGFβ1 in some experiments but it was more reliable and efficient in the presence of TGFβ1. 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 μg/ml of P4 (Fig.1C).

It has been reported that induced human $FoxP3^+$ T cells with TGFβ1 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 to IL-2-treated T cells, P4+IL-2-treated T cells displayed a potent suppressive activity. Similarly, P4 and TGFβ1-treated T cells displayed a greater suppressing activity on the proliferation of target ($CD4^+CD25^-$) T cells compared to TGFβ1 alone-treated T cells (Fig.1D). RU486, an antagonist for several different progesterone receptors, fully suppressed the P4-induced expression of the FoxP3 protein and transcription of the *FoxP3* gene (Fig.1E and F).

Adult peripheral blood naïve T cells are resistant to P4-induced conversion into $FoxP3^+$ T cells

It has been previously reported that CB is a better source of Treg progenitors compared to PB (38). We examined if CB T cells are different from PB T cells in response to P4. Compared to the highly sensitive CB T cells, adult PB naïve 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 naïve T cells during allogeneic responses between T cells and antigen presenting cells (APC), we co-cultured naïve $CD4^+$ 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 naïve T cells were again poorly responsive. CB APC were more potent than adult APC in induction of P4-induced $FoxP3^+$ T cells (Fig.2B).

Next, we asked if CB naïve T cells would remain responsive to P4 even after previous antigen priming events. To address this, CB T cells were cultured first with or without P4 and then recultured with P4 and/or TGFβ1 (Fig.3). P4 was unable to induce FoxP3 expression once the CB naïve T cells were pre-activated. Initial exposure to P4 was critical to induce $FoxP3^+$ T cells at high levels by P4 upon subsequent activation with TGFβ1 alone or P4 and TGFβ1. 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 TGFβ1 if they were 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 naïve cells, contain a significant memory ($CD45RA^-CD45RO^+$) cell population, suggesting a history of ongoing antigen priming events in fetuses (Fig.4A). This can be induced preferentially by allogeneic immune responses between fetal T cells and maternal APC or fetal APC presenting maternal antigens. Interestingly, 30–40% of these memory T cells were $FoxP3^+$ T cells (Fig.4A and B). This is considered very high compared to the low frequency (~10%) of $FoxP3^+$ T cells among adult PB memory T cells (Fig.4B). While ~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 antigen-primed and assumed the memory T cell phenotype. These memory type CB $FoxP3^+$ T cells expressed CD49f (integrin α6) and CD58 (LFA-3), the antigens typically associated with antigen-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 antigen-primed without exogenous P4 (but in the presence of TGFβ1) were mostly T cells of intermediary $CD45RO^-CD45RA^-$ phenotype, suggesting that P4 has a profound regulatory effect on expression of the naïve and memory cell-associated antigens 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 to 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 TGFβ1. However, they are different in that IL-2 promotes FoxP3$^+$ T cells and suppresses Th17 cell differentiation, while 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 naïve CB CD4$^+$ T cells in a Th17 cell induction condition in the presence and absence of P4. P4 dramatically reduced the conversion of naïve T cells into Th17 cells (Fig.6A). 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, 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

STAT5A/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 STAT5A/B in the CB T cells induced by IL-2 (Fig.7A). P4 by itself, however, did not induce phosphorylation of STAT5A/B. We examined also the activation of STAT3 because this molecule is central to transmit signals to induce Th17 cells and suppress Tregs. Upon stimulation with IL-6, T cells cultured with P4 had decreased phosphorylation of STAT3 compared to 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 up- or down-regulated by P4 either in the presence or absence of exogenous TGFβ1 (Fig.8A and Supplemental Fig.1A and B). PB T cells were relatively unresponsive with only 30–70 genes regulated by P4. IL-6 receptor (IL-6R) expression was greatly down-regulated 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 and C). 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 transcription factor-like 1 (LZTFL1) (43), methyltransferase like 7A (METTL7A), hypermethylated in cancer 1 protein (HIC1), growth factor independent 1 transcription repressor (GFI1) (44), suppressor of cytokine signaling 1 (SOCS1), SOCS2, SOCS3, CC

chemokine ligand 5 (CCL5), 2',5'-oligoadenylate synthetase 1 (OAS1), and interferon-induced protein with tetratricopeptide repeats 3 (IFIT3). Among these, SOCS1 and SOCS2 were down-regulated, while others are up-regulated. A more extensive list can be found in Supplemental Figure 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 naïve T cell differentiation into Tregs expressing FoxP3. Interestingly, CB T cells were highly responsive to P4 in this process while adult PB T cells were largely unresponsive to P4. Second, while P4 promotes the differentiation of naïve 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 antigen 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 so far 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 a potent enhancing effect on TGFβ1-induced generation of $FoxP3^+$ T cells. Reciprocally, retinoic acid has a suppressive effect on generation of Th17 cells. Also, P4 is implicated in induction and expansion of $CD4^+CD25^+$ Treg cells in the uterus 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 naïve 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 antigen presenting cells of CB provide signals conducive for generation of Tregs. When CB T cells are antigen-primed by allogeneic APC, CB APC were significantly more efficient in inducing $FoxP3^+$ T cells compared to PB APC. All of the characteristics of CB T cells and APC 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 placenta tissue (3, 52). Thus, up to ~3000 ng/ml of 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 TGFβ1, 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 fetus. The difficulty of adult T cells to become Tregs in response to P4, however, suggests that the P4 function is more important in fetus and newborns than pregnant females. Therefore, a hypothetical role for P4-induced induction of regulatory T cells 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 the T cell differentiation into $FoxP3^+$ T cells but restrains the process 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 for 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 to be 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 up or down-regulating genes, while 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 up-regulated with P4, TNFSF13B/BAFF is known to expand $CD4^+CD25^+Foxp3^+$ Tregs (56). EBI3, 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 nicotinamide adenine dinucleotide (NAD) (60), was increased by P4. Some of the genes such as *OAS1*, *RGS1* and *TNF13B*, highly induced in response to P4, were among the genes induced in Tregs derived from fetal stem and progenitor cells (25). Roles of the P4-regulated molecules in control of T cell differentiation and function remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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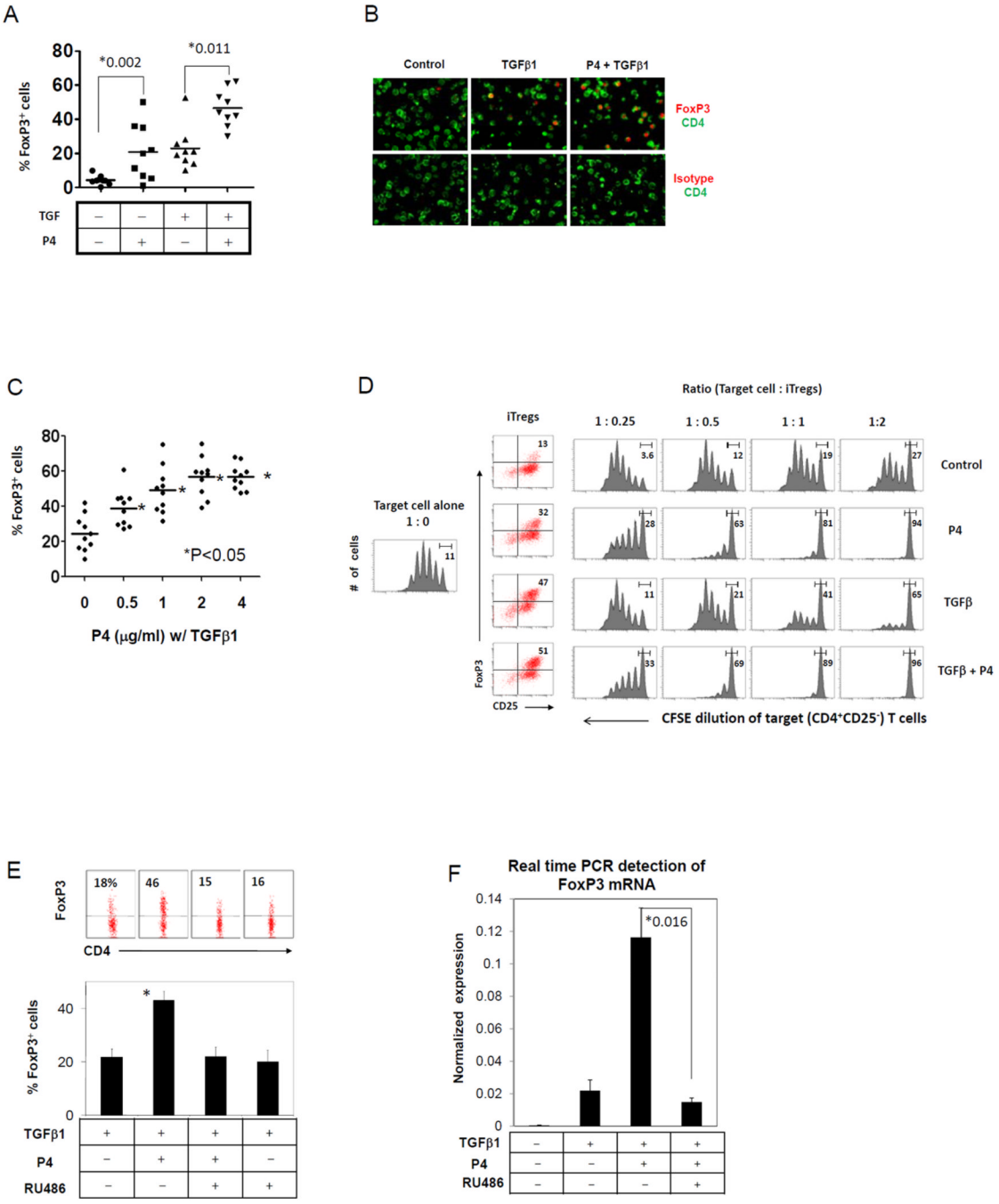

Fig. 1. P4 drives the differentiation of CB CD4$^+$ T cells into highly suppressive Tregs

(A) Treg-depleted CB fetal (CD4$^+$CD25$^-$) T cells were activated with beads coated with antibodies to CD3 and CD28, P4 (2 μg/ml) and IL-2 for 7 days, and frequencies of FoxP3$^+$ T cells were determined by flow cytometry. TGFβ1 was added as indicated. (B) Microscopic detection of FoxP3 expression in CB T cells stimulated in the presence of P4 and/or TGFβ1. (C) A dose-dependent response of T cells to P4 in FoxP3 induction in the presence of TGFβ1. (D) The suppressive function of control and P4-treated CB T cells on the proliferation of CFSE-labeled CB CD4$^+$CD25$^-$ naive T cells were examined. To prepare suppressors, CB naïve (CD4$^+$CD25$^-$) T cells were cultured in the presence of IL-2 (control), or IL-2 and P4 (P4) for 6–7 days. TGFβ1 was added as indicated. The suppressors were

cultured with CFSE-labeled target T cells for 5 days and the CFSE intensity of target cells was determined by flow cytometry. 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 Fig. 1A (n=9), 1C (n=10), 1E (n=7), and 1F (n=3). *Significant differences between the two groups with P values or from the TGFβ1-alone control group.

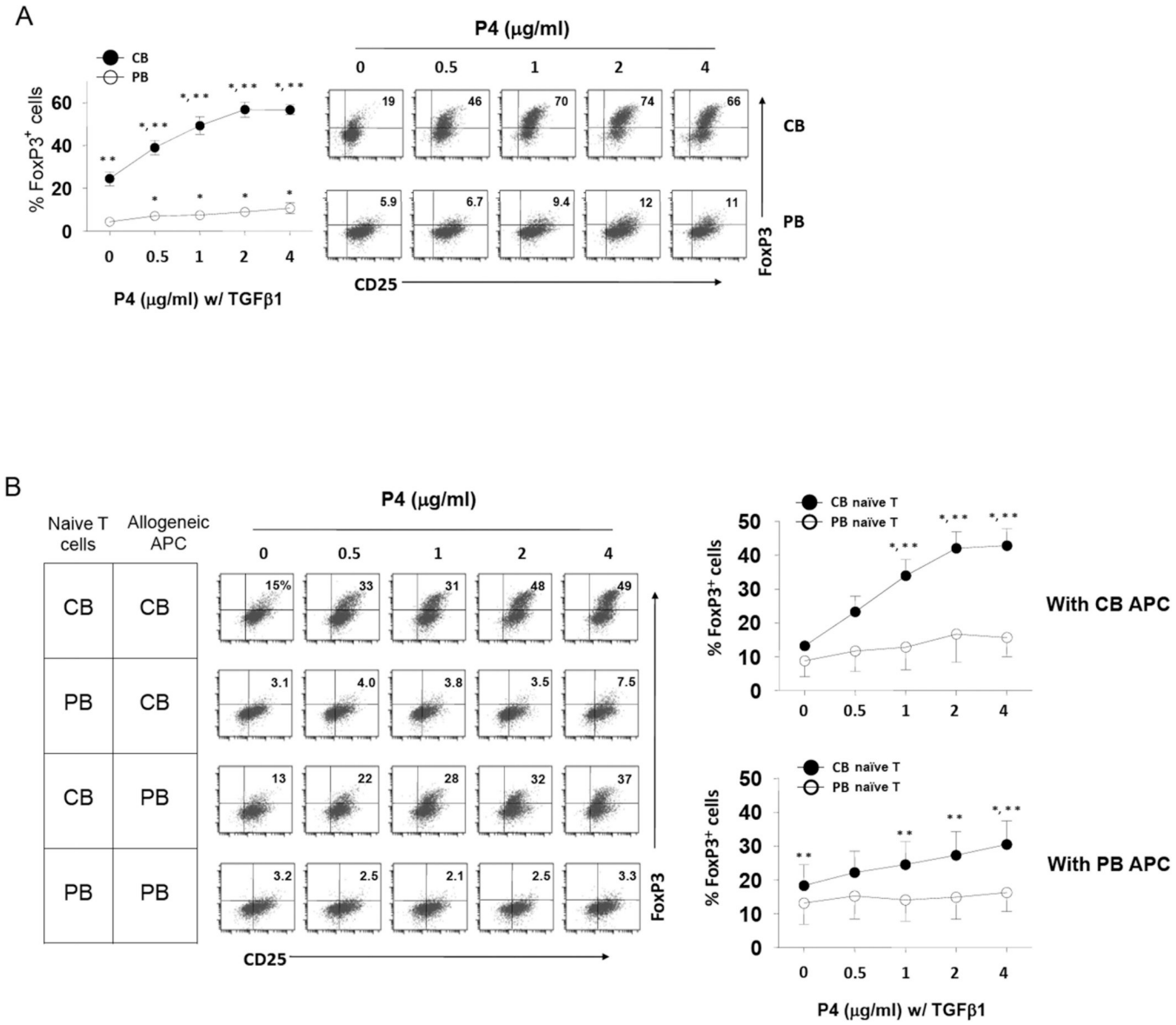

Fig. 2. CB, but not adult PB, naïve T cells differentiate into FoxP3$^+$ Tregs in response to P4

(A) CB naive T cells and adult PB naïve CD4$^+$ T cells were compared for their response to P4. T cells were activated with beads coated with antibodies to CD3 and CD28, P4 (2 μg/ml) and IL-2 for 7 days. TGFβ1 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 antigen priming-induced differentiation of CB T cells into FoxP3$^+$ T cells. Naïve CD4$^+$ T cells isolated from the indicated sources were co-cultured with allogeneic APC (irradiated mononuclear cells) obtained from CB or PB for 7 days in the presence of indicated concentrations of P4 (n=4–6). Significant differences from the control point (0 μg/ml)* or from PB**.

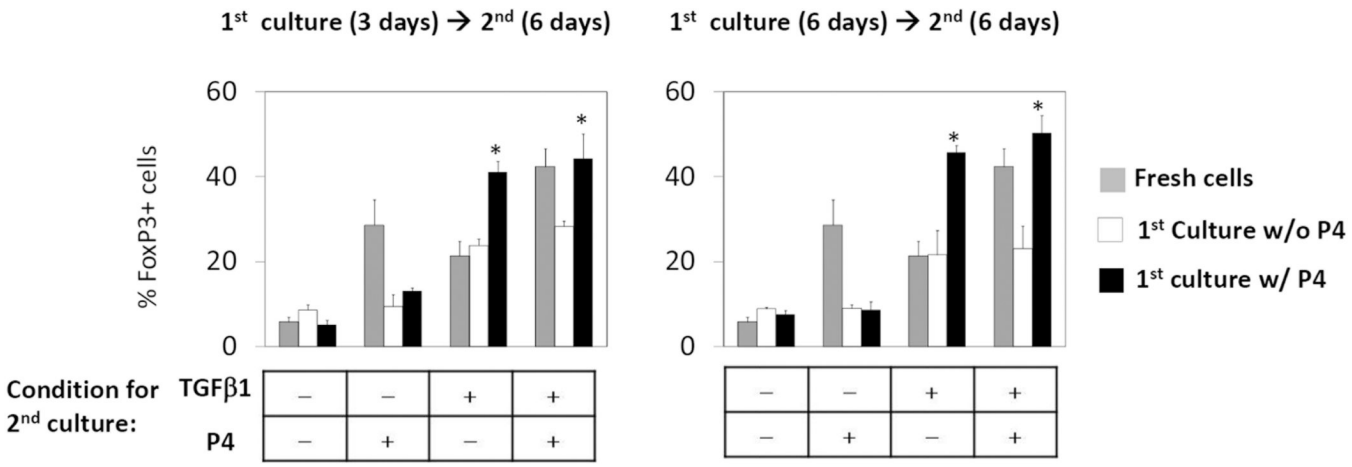

Fig. 3. Impact of P4 on stability of FoxP3 expression in T cells

CB naïve $CD4^{+}CD25^{-}$ T cells were activated with beads coated with antibodies to CD3 and CD28, P4 (2 μg/ml) and IL-2 for 3 or 6 days and then recultured with P4 and/or TGFβ1 for 6 days. Anti-CD3/28 and IL-2 were used to activate the T cells. FoxP3 expression was measured following the second culture. Combined data from 3–4 experiments are shown. *Significant differences from the respective control groups (no P4).

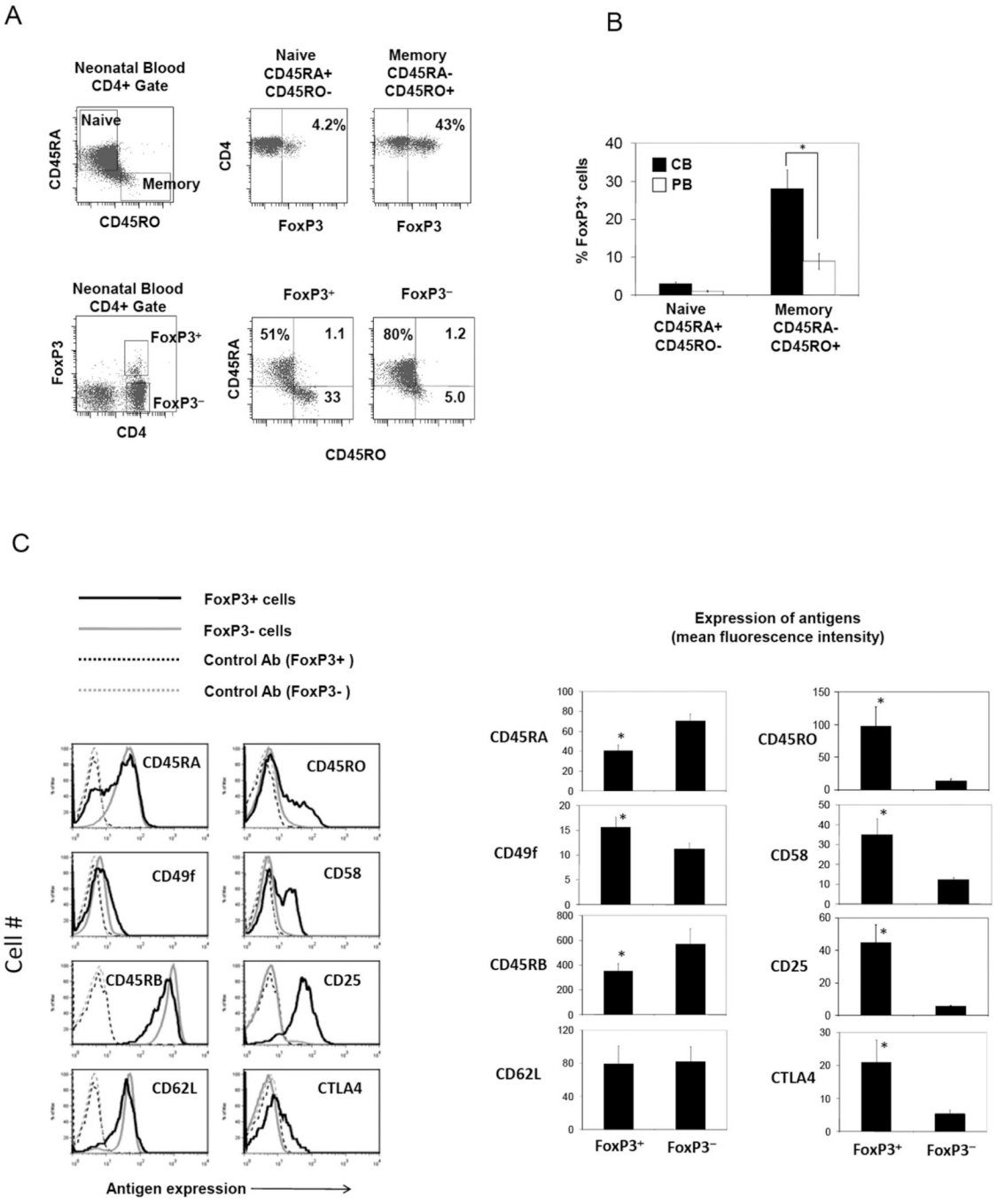

Fig. 4. The CB Treg compartment is enriched with memory type FoxP3$^+$ T cells

(A) Frequencies of naïve versus memory FoxP3$^+$ and FoxP3$^-$ T cells in fresh CB. (B) Comparison of CB and adult PB in frequencies of naïve 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.

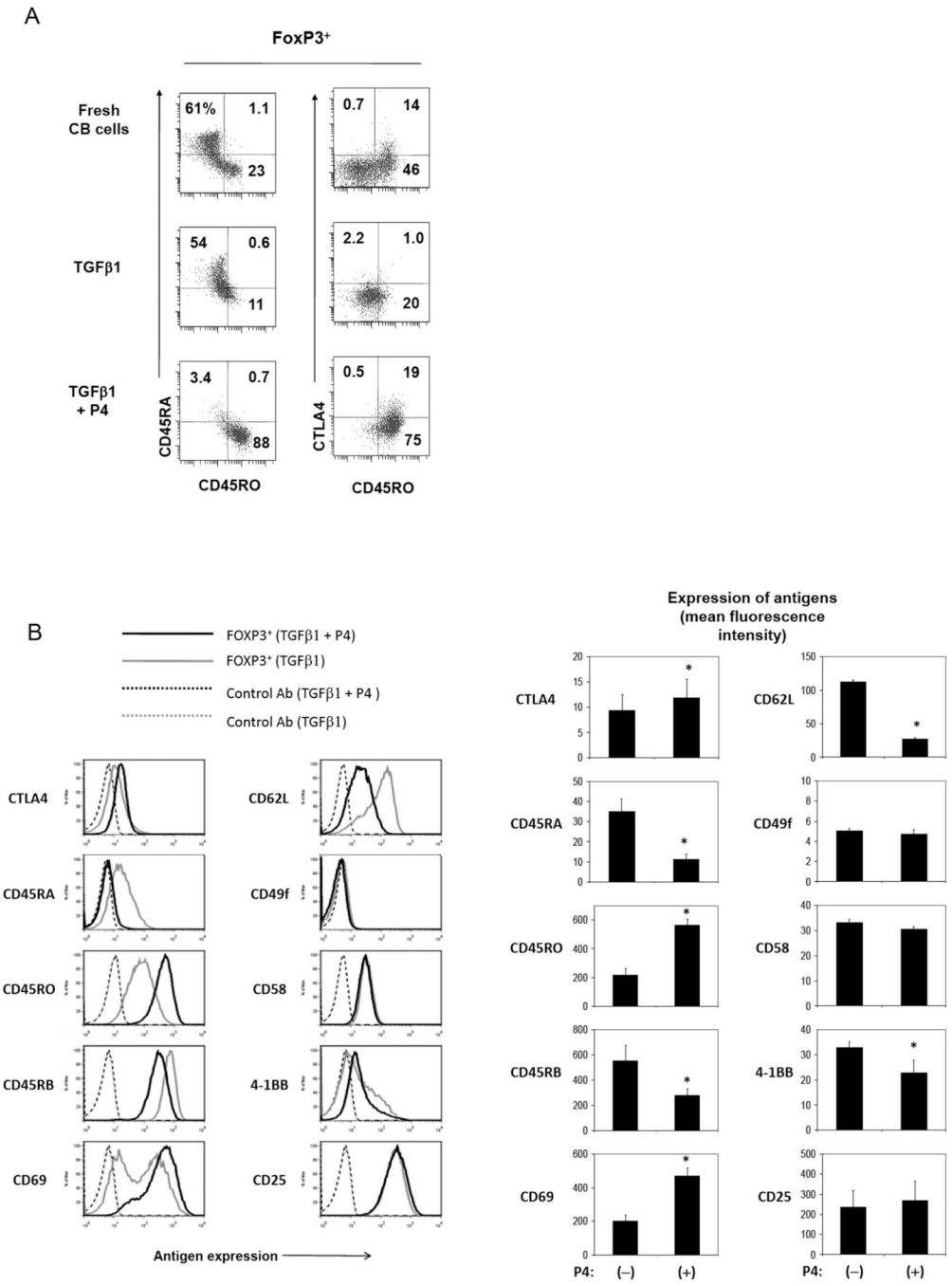

Fig. 5. P4 promotes the induction of memory type FoxP3$^+$ T cells

(A) Emergence of CD45RA$^-$ CD45RO$^+$ FoxP3$^+$ T cells from CB naïve CD4$^+$ T cells in response to P4 in vitro. (B) Expression of various surface antigens by the P4-induced or control iFoxP3$^+$ T cells. CB naïve CD4$^+$CD25$^-$ T cells were activated with beads coated with antibodies to CD3 and CD28, P4 (2 μg/ml) and IL-2 for 7 days. Combined data for surface antigens (n=4) expressed by control and P4-induced FoxP3$^+$ T cells are shown in a graph form. *Significant differences from the control group (no P4).

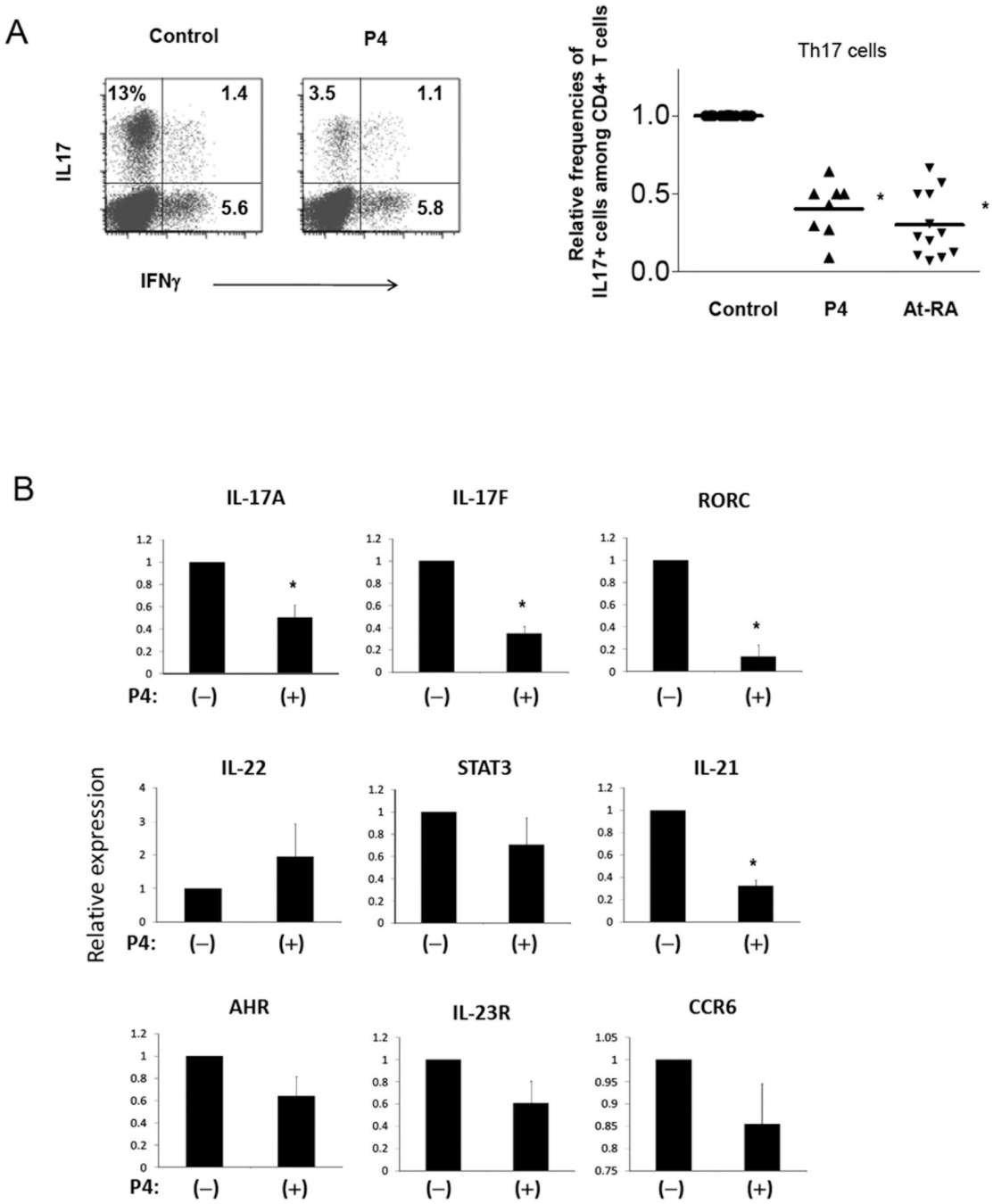

Fig. 6. P4 is an effective suppressor of T cell differentiation into Th17 cells

Treg-depleted CB T cells were cultured in a Th17 cell-induction condition (anti-CD3/CD28 beads or PHA in the presence of IL-23, IL-1β, TGFβ1, anti-IL-4, and anti-IFNγ) for 7 days with or without P4 (2 μg/ml). The relative frequencies of Th17 cells and Th1 cells (A) and expression of Th17-associated genes at the RNA level determined with a real-time PCR method (B) is shown. The combined data in panel A were obtained from 8–12, and the data in panel B from 3–5, independent experiments. *Significant differences from the control group.

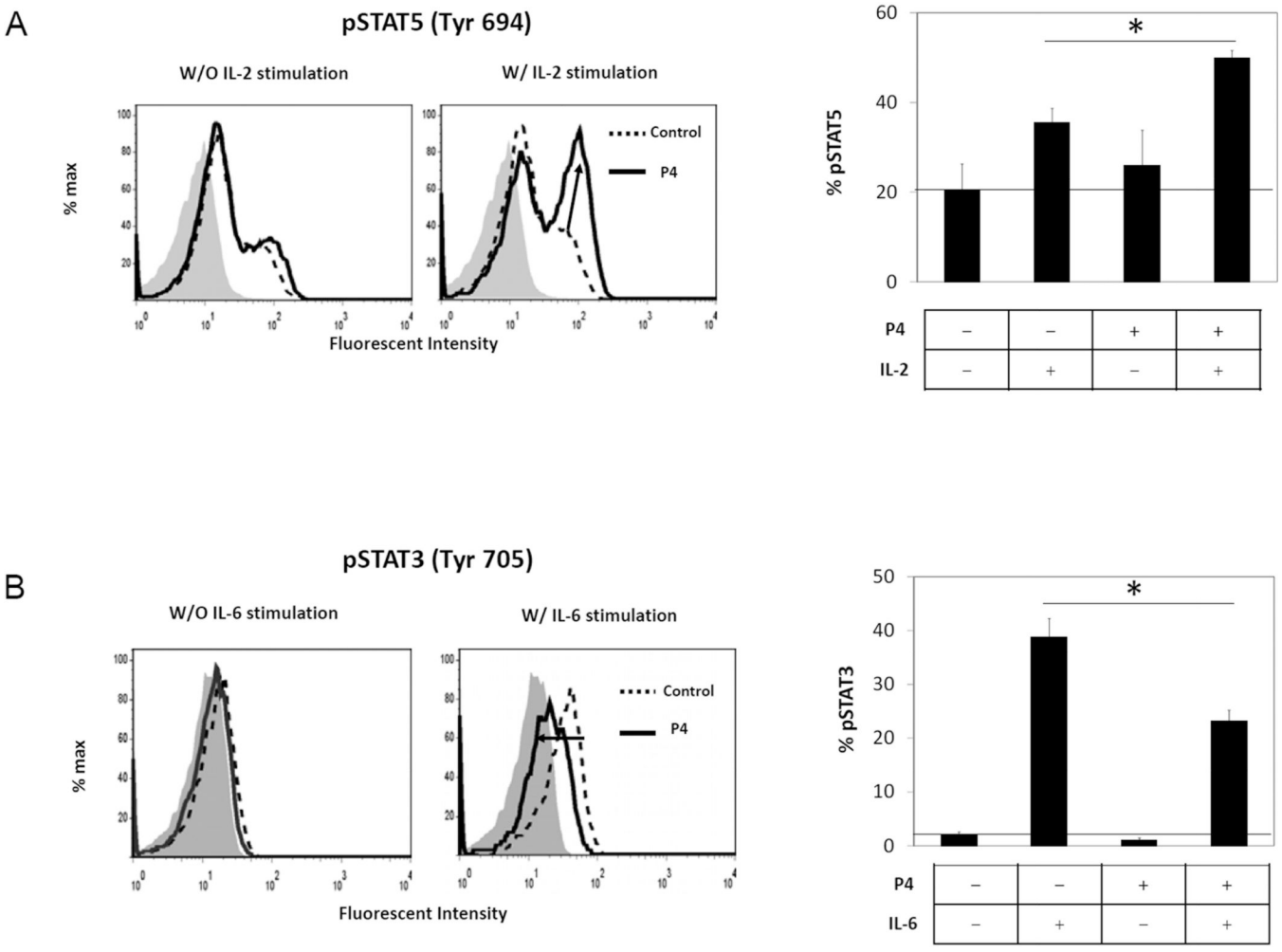

Fig. 7. P4 enhances STAT5 activation in response to IL-2 while it suppresses STAT3 activation in response to IL-6

CB $CD4^{+}CD25^{-}$ T cells were activated with anti-CD3/CD28 beads in the presence or absence of P4 for 20 hours. 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 anti-STAT5 (pY694) or anti-STAT3 (pY705) antibody respectively. A control antibody was employed 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.

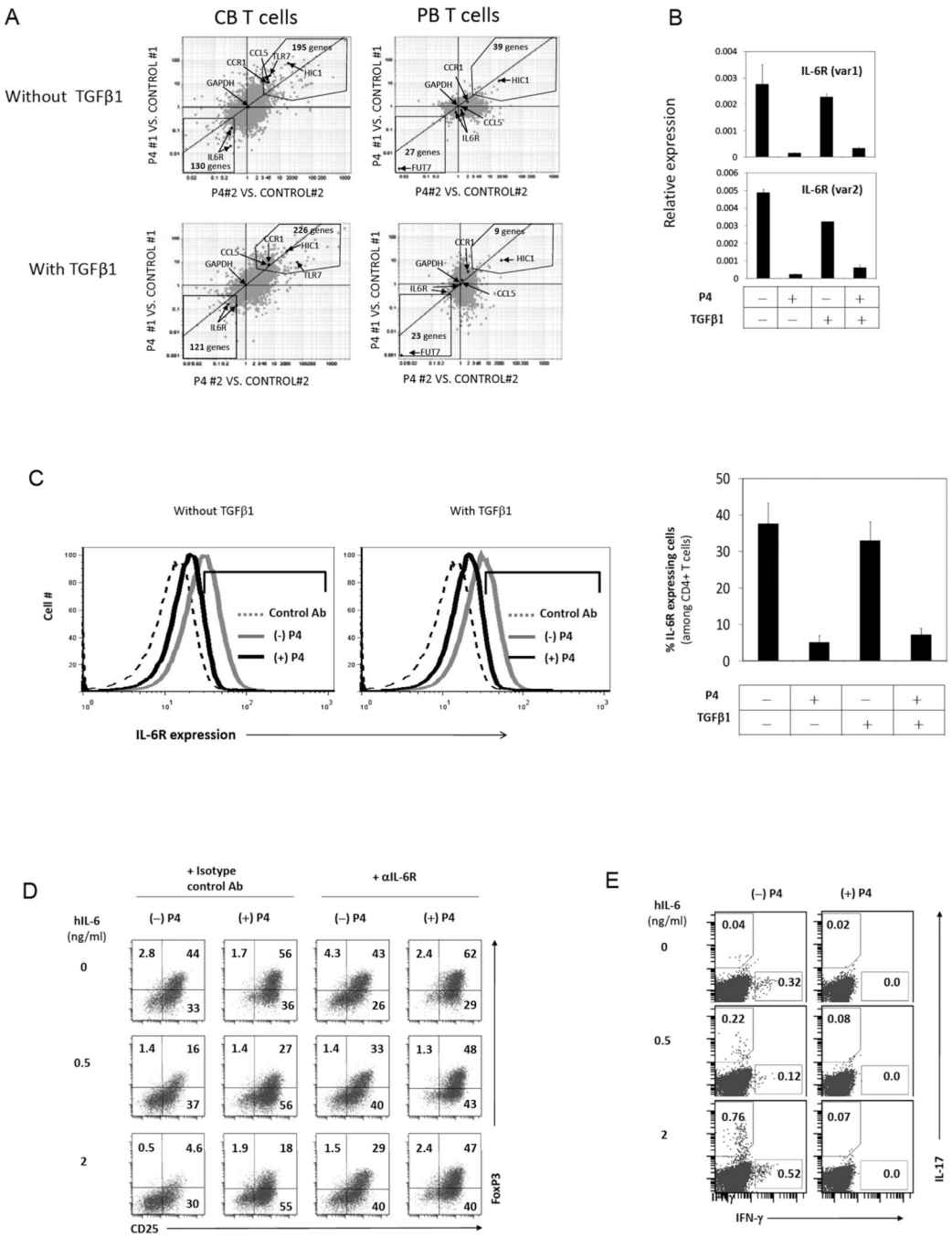

Fig. 8. The function of P4 in induction of Tregs and suppression of Th17 cells is mediated through induction or suppression of distinct groups of genes

(A) Multiplots showing P4-regulated genes. CB or PB naive T cells were activated with beads coated with antibodies to CD3 and CD28, P4 (2 μg/ml), and IL-2 for 5 days for the microarray study. The multiplots show groups of genes reproducibly up- or down-regulated in CB or PB T cells by P4. More detailed information is shown in Supplemental Figure 1. Expression of IL-6R is blocked by P4 in T cells at the RNA level (B) and the surface protein level (C). P4 promotes the generation of FoxP3^{+} T cells (D) but suppresses the induction of Th17 cells (E) in the presence of hIL-6. A representative data set out of 3 separate experiments is shown.