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Felicità Baratelli, Ying Lin, Li Zhu, Seok-Chul Yang, Nathalie Heuzé-Vourc'h, Gang Zeng, Karen Reckamp, Mariam Dohadwala, Sherven Sharma and Steven M. Dubinett

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Prostaglandin E2 Induces FOXP3 Gene Expression and T Regulatory Cell Function in Human CD4+ T Cells

Felicita Baratelli, Ying Lin, Li Zhu, Seok-Chul Yang, Nathalie Heuzé-Vourc'h, Gang Zeng, Karen Reckamp, Mariam Dohadwala, Sherven Sharma, and Steven M. Dubinett

Naturally occurring CD4+CD25+ regulatory T cells (T reg) are pivotal in suppressing immune responses and maintaining tolerance. The identification of molecules controlling T reg differentiation and function is important in understanding host immune responses in malignancy and autoimmunity. In this study we show that PGE2 enhances the in vitro inhibitory function of human purified CD4+CD25+ T reg cells. Moreover, PGE2 induces a regulatory phenotype in CD4+CD25+ T cells. PGE2-treated T cell-mediated inhibition of anti-CD3-stimulated lymphocyte proliferation did not require cell contact. Phenotypic analysis revealed that PGE2 diminished CD25 expression in both CD4+CD25dim T cells and CD4+CD25bright T reg cells. PGE2 exposure induced the T reg cell-specific transcription factor forkhead/winged helix transcription factor gene (FOXP3) in CD4+CD25+ T cells and significantly up-regulated its expression in CD4+CD25+ T reg cells. Similarly, 24-h incubation with supernatants from cyclooxygenase-2-overexpressing lung cancer cells that secrete high levels of PGE2 significantly induced FOXP3 in CD4+CD25+ T cells. Finally, PGE2 up-regulated FOXP3 at both mRNA and protein levels and enhanced FOXP3 promoter activity. This is the first report indicating that PGE2 can modulate FOXP3 expression and T reg function in human lymphocytes. The Journal of Immunology, 2005, 175: 1483–1490.

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Abbreviations used in this paper: T reg cell, regulatory T cell; AS, antisense; COX-2, cyclooxygenase-2; CV, control empty vector; FOXP3, forkhead/winged helix transcription factor gene; NSCLC, nonsmall cell lung cancer; S, sense.

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Louis, MO) was added, and a mouse IgG (10 µg/ml; Sigma-Aldrich) was used as a control Ab. T cell activation was performed by incubation of both CD4+CD25+ T cells and CD4+CD25− T reg cells in plate-bound anti-CD3 (1 µg/ml)-coated, 96-well plates (BD Biosciences) from 24 to 120 h. Alternatively, T cells were activated in the presence of PMA (20 ng/ml; Sigma-Aldrich) and ionomycin (1 µg/ml; Sigma-Aldrich).

Flow cytometric analysis
CD4+CD25− T cells and CD4+CD25+ T reg cells, pretreated with or without PGE2, and cultured with or without anti-CD3 stimulation, were resuspended in PBS/2% FBS and labeled with human anti-CD3 PE (Miltenyi Biotec) and human anti-CD25 FITC Abs or with control isotypic (all from BD Biosciences) at the indicated time points. Acquisition was performed using a Life Science Research flow cytometer or a SCAN X instrument (BD Biosciences) using CellQuest software (BD Biosciences) at University of California-Los Angeles Jonsson Comprehensive Cancer Center Flow Cytometry Core Facility. Data analysis was performed with CellQuest software.

In vitro proliferation assay
T cell proliferation was assessed by BrdU incorporation using a BrdU ELISA colorimetric assay (Roche). Briefly, magnetic bead-purified human CD4+CD25− T cells or CD4+CD25+ T reg cells were pretreated with PGE2 (13 or 26 µM) for 24 h. PGE2 was removed by washing before starting the proliferation assay. To rule out the possibility that residual PGE2 would be transferred to the second culture, we performed preliminary experiments in which PGE2 was pretreated with PGE2 (26 µM; Cayman Chemical) catalogue no. 14010) for 12–18 h. The presence of PGE2 in the supernatant was then assessed by a specific enzyme immunoassay (PGE2, EIA kit; Cayman Chemical) after cell washing. The latter procedure was determined to be effective in removing >99% of the added PGE2 (data not shown). Both CD4+CD25− and CD4+CD25+ T cell populations, with or without PGE2 pretreatment, were then cultured at a 1:1 ratio with autologous CD4+CD25− responder T cells (1 × 105) in anti-CD3 plate-bound, 96-well plates. In some experiments IL-2 (Proleukin; biological activity, 18 × 104 IU/1 ml; Chiron) or IL-7 (biological activity, 2 × 104 IU/ml; PeproTech) was added to the cells. The concentrations of IL-7 and IL-2 used in the proliferation assays (2 ng/ml equivalent to 4 U/ml and 20 IU/ml corresponding to 1.56 ng/ml, respectively) were determined based on previous reports (26, 27) and our own preliminary studies. In four independent experiments, the T cell proliferations expressed as OD values of BrdU incorporation on day 5 of culture were 0.95 ± 0.3 in the presence of IL-2 (20 IU/ml) and 0.93 ± 0.06 in the presence of IL-7 (2 ng/ml). Thus, the concentrations of IL-2 and IL-7 used were comparable based on the capacity of both cytokines to induce a similar degree of proliferation of CD3-activated CD4+ T cells in vitro. After 5 days in culture, cells were pulsed with BrdU and 4 h later were assessed for BrdU incorporation. To analyze cell contact-independent inhibition, 3 × 105 untreated or PGE2-treated CD4+CD25− T cells and untreated or PGE2-treated CD4+CD25+ T reg cells were separated from mitomycin C (50 µg/ml; Sigma-Aldrich)-treated, allogeneic-dendritic cell-stimulated CD4+CD25− responder T cells (3 × 105) by 3-µm Transwell inserts (Costar; Corning) in 24-well plates. T cell proliferation was then measured after a 5-day culture period by analysis of BrdU incorporation. Results are expressed as the percentage of proliferation of responder T cells alone.

Real-time PCR for FOXP3
For quantitative real-time analysis, RNA was extracted, and cDNA was prepared with a kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. Human FOXP3 mRNA expression was quantified using the SYBR Green quantitative PCR kit (Finnzymes) in the iCycler (Bio-Rad) and was corrected with human β-actin housekeeping control amplifications. Amplification was conducted in a total volume of 20 µl for 40 cycles of 15 s at 95°C, 20 s at 60°C, and 30 s at 72°C. Samples were run in triplicate, and their relative expression was determined by normalizing the expression of each target to β-actin and then comparing this normalized value to the normalized expression in a reference sample to calculate a fold change value.

For FOXP3, primers were: forward, 5′-CAA GTC CAA CAA CAT CGG AC-3′; and reverse, 5′-ATT GAG TGG TGT CCG TTG CT-3′. FOXP3 primers were synthesized by Integrated DNA Technologies. For β-actin, primers were: forward, 5′-GAT GAG TGG CAT GCG TAT G-3′; and reverse, 5′-CACC TCT CCG TTG CAG G-3′.

FOXP3 promoter cotransfection of Jurkat T cells and dual luciferase assay
A 350-bp fragment (FOXP3-SFUT) containing the FOXP3-scFv gene intron 0 upstream from the initiation codon, located within the 65,825–65,475 bp region of human chromosome X, multiple clone map pII.23, was inserted into a freely luciferase reporter vector (pGL3-basic; Promega) as previously described (28). FOXP3-SFUT and Renilla luciferase gene CV pRL-TK Renilla were then cotransfected into Jurkat T cells using the SuperFect Transfection Reagent (Qiagen). PGE2 (13 μM), PMA (20 ng/ml) plus ionomycin (1 µg/ml) (PI), and PMA, ionomycin, and PGE2 were added separately to Jurkat T cells 24 h after transfection for an additional 18 h. Firefly and Renilla luciferase activities were measured using the dual luciferase reporter assay system (Promega) and a single-sample luminometer (Becton Dickinson; GEM Biomedical). Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase.

Western blot analysis for FOXP3
FOXP3 protein expression was analyzed in the lysate of Jurkat T cells (5.0 × 105 cells). The protein concentration in the cell lysate was determined using a bicinchoninic acid assay (Pierce). Protein-normalized aliquots of the cell lysate (25 µg) were electrophoresed on an 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. FOXP3 was immunodetected with 1/5000 rabbit anti-human FOXP3 polyclonal Ab (Abcam), followed by incubation with 1/500 HRP-conjugated donkey anti-rabbit Ig (Santa Cruz Biotechnology). Immunoblots were developed using an ECL detection system (Supersignal West Pico chemiluminescence; Pierce), followed by autoradiography. Equal protein loading was confirmed by immunodetecting the membranes with anti-GAPDH Ab (Advanced Immunocoolaborative). Relative protein quantification was determined by computerized densitometric analysis using Scion Image software (version 1.62c).

Statistics
The p values were calculated using unpaired Student’s t test. Values ≤0.05 were considered significant.

Results
PGE2 enhances CD4+CD25+ T regulatory function and confers T regulatory activity in CD4+CD25− T cells
Previous studies have focused on the direct effect of PGE2 in mediating suppression of lymphocyte proliferation, highlighting the role of calcium-dependent mechanisms (29, 30). In the current study we first investigated whether exogenous PGE2 was able to augment the suppressive activity of CD4+CD25+ T reg cells. Purified CD4+CD25+ T reg cells demonstrated significant enhancement of their inhibitory function after incubation with PGE2 for 24 h in vitro (Fig. 1A). Treatment of CD4+CD25+ T reg cells with escalating doses of PGE2 (13 or 26 µM) inhibited T cell proliferation in a dose-dependent pattern (Fig. 1A). The T cell proliferations, expressed as OD values of BrdU incorporation, obtained from seven different donors were 1.04 ± 0.17 (CD4+CD25−), 0.68 ± 0.16 (CD4+CD25+), and 0.34 ± 0.14 (CD4+CD25+ + PGE2; 26 µM). During the 5-day culture period of the assay, we examined the T cell proliferative response by light microscopy. Wells containing stimulator T cells not treated with PGE2 displayed numerous homogeneously distributed cell clusters, indicative of ongoing proliferation. In contrast, wells containing stimulator T cells pretreated with PGE2 consistently showed significantly fewer proliferating clusters (data not shown). The OD values of BrdU incorporation reflected the above observations. Accumulating evidence indicates that T cells with regulatory features can be generated in vitro (6–13). In the present study, preincubation of CD4+CD25− T cells with PGE2 (13 or 26 µM) conferred regulatory T cell function (Fig. 1B). In fact, the PGE2-treated CD4+CD25− stimulator cells inhibited the CD3-activated proliferation of autologous CD4+CD25+ responder T cells. The levels of inhibition demonstrated by CD4+CD25− T cells exposed to PGE2 were similar to those induced by untreated CD4+CD25− T
reg cells (Fig. 1B). The T cell proliferations, expressed as OD values of BrdU incorporation, obtained from three different donors were 1.26 ± 0.39 (CD4$^+$CD25$^+$) and 0.65 ± 0.40 (CD4$^+$CD25$^+$ plus PGE$_2$ (26 μM)). Addition of IL-7 (2 ng) partially overcame the PGE$_2$-mediated suppression and reversed the CD4$^+$CD25$^+$ T reg cell-mediated inhibition of proliferation (Fig. 1C). The T cell proliferations, expressed as OD values of BrdU incorporation, obtained from three different donors were 0.93 ± 0.06 (CD4$^+$CD25$^+$), 0.72 ± 0.17 (CD4$^+$CD25$^+$ plus PGE$_2$ (26 μM)), 0.89 ± 0.07 (CD4$^+$CD25$^+$), and 0.69 ± 0.22 (CD4$^+$CD25$^+$ plus PGE$_2$ (26 μM)). In contrast, IL-2 (20 IU/ml) did not overcome the PGE$_2$-induced or the CD4$^+$CD25$^+$ T reg cell-induced suppression (Fig. 1D). The T cell proliferations, expressed as OD values of BrdU incorporation, obtained from three different donors were 0.95 ± 0.29 (CD4$^+$CD25$^+$), 0.46 ± 0.25 (CD4$^+$CD25$^+$ plus PGE$_2$ (26 μM)), 0.64 ± 0.36 (CD4$^+$CD25$^+$), and 0.31 ± 0.16 (CD4$^+$CD25$^+$ plus PGE$_2$ (26 μM)). In assays that directly assessed CD4$^+$CD25$^+$ T reg cell proliferation, both IL-7 (≥2 ng/ml) and a high concentration of IL-2 (≥100 IU/ml) demonstrated the capacity to induce proliferation of T reg cells (data not shown) in agreement with previous reports (31, 32). Interestingly, when CD4$^+$CD25$^+$ responder T cells were separated by Transwell inserts from PGE$_2$-treated stimulator cells (CD4$^+$CD25$^+$ or CD4$^+$CD25$^+$), both PGE$_2$-treated populations maintained the capacity to inhibit T cell proliferation (Fig. 1E). These data taken together suggest that the PGE$_2$-induced T regulatory function does not require cell contact. We have previously found that PGE$_2$ is a potent inducer of lymphocyte IL-10 gene transcription (15), and PGE$_2$ has also been reported to increase TGF-$eta$ expression (33–35). However, neither anti-IL-10 nor anti-TGF-$eta$ neutralizing Abs were able to reverse the PGE$_2$-induced inhibitory activity mediated by CD4$^+$CD25$^+$ or CD4$^+$CD25$^+$ T reg cells (data not shown). These findings suggest the contribution of additional soluble factors other than IL-10 or TGF-$eta$ to PGE$_2$-induced cell-contact independent T regulatory cell function.
Down-regulation of CD25 expression in PGE$_2$-treated CD4$^+$ T cells

Because T reg cells are identified as a CD4$^+$CD25$^{bright}$ T cell subset (36), we analyzed whether the PGE$_2$-treated CD4$^+$CD25$^{dim}$ starting population acquired the same phenotype of naturally occurring, thymic-derived CD4$^+$CD25$^{bright}$ T reg cells. As shown in Table I, CD25 was differentially expressed in magnetic bead-purified CD4$^+$CD25$^{dim}$ and CD4$^+$CD25$^{bright}$ T cells. Indeed, with (Fig. 2A and Table II) or without PGE$_2$ preincubation (data not shown), CD4$^+$CD25$^{dim}$ up-regulated CD25 as early as 24 h after stimulation with anti-CD3 plate-bound Ab (Fig. 2A). These T cells maintained high levels of CD25 expression throughout a 96-h culture period (Fig. 2A). However, when anti-CD3 Ab-activated T cells were pretreated with PGE$_2$, CD25 expression declined in both CD4$^+$CD25$^{dim}$ T cells (Fig. 2B, bottom left, and Table II) and CD4$^+$CD25$^{bright}$ T reg cells (Fig. 2B, bottom right, and Table III), compared with their counterparts without PGE$_2$ exposure (Fig. 2, B and C, upper panels, and Tables II and III). Despite decreased CD25 expression, suppressive T reg function was maintained, as shown in Fig. 1. Other markers indicative of the T reg phenotype, including glucocorticoid-induced TNFR family-related protein, CTLA-4, human latency-associated peptide of TGF-$eta_1$, lymphocyte activation Ag 3, PD-1, CD103, CD122, and CCR4, were either not altered or were down-regulated by PGE$_2$ (data not shown).

PGE$_2$ induces FOXP3 in CD4$^+$CD25$^-$ T cells and up-regulates its expression in CD4$^+$CD25$^+$ T reg cells

Because FOXP3 is specifically expressed in naturally occurring T reg cells and programs their development and function (1–5), we determined whether PGE$_2$ had an impact on the induction of FOXP3 gene expression in both CD4$^+$CD25$^+$ T reg and CD4$^+$CD25$^-$ T cells. As expected, purified, peripheral blood-derived, CD3-activated CD4$^+$CD25$^+$ T reg cells expressed high levels of FOXP3 mRNA compared with CD4$^+$CD25$^-$ T cells (Fig. 3A). Furthermore, a 24-h preincubation with PGE$_2$ (26 M) resulted in >4-fold up-regulation of FOXP3 (Fig. 3A). When CD4$^+$CD25$^-$ T cells were cultured under the same stimulatory conditions in the presence of PGE$_2$ (26 M), FOXP3 mRNA expression was also significantly induced, consistent with the acquisition of a T reg cell suppressive function (Fig. 3B). Thus, PGE$_2$ facilitated the differentiation of CD4$^+$CD25$^-$ T cells into a T cell population endowed with inhibitory properties and FOXP3 mRNA expression.

Both preclinical and clinical studies have suggested that T reg cells play a role in suppressing the host immune response to malignancy. In particular, recent reports have demonstrated enhanced T reg cell activity in cancer patients (17–22). Among the malignancies associated with augmented T reg cell function are tumors known to overexpress COX-2 and secrete high levels of PGE$_2$ (17–22). Thus, to determine the importance of tumor-derived PGE$_2$, we used COX-2 gene-modified NSCLC cells as an experimental model for the tumor site, which is often enriched for PGE$_2$. For this purpose we genetically modified NSCLC cell lines to express COX-2 S, COX-2 AS, or empty CV, as described in Materials and Methods and as we have previously reported (25). Using this in vitro model we preincubated CD4$^+$CD25$^-$ T cells with tumor supernatant from H157 COX-2-gene modified NSCLC cell lines. FOXP3 gene expression was quantified in CD4$^+$CD25$^-$ cells after 24-h activation with anti-CD3 plate-bound Ab. A 24-h exposure to H157 COX-2 S supernatant significantly induced FOXP3 gene expression in CD4$^+$CD25$^-$ T cells. In contrast, COX-2 AS and CV supernatant had no effect on FOXP3 gene expression. Addition of anti-PGE$_2$ Ab to COX-2 S supernatant blocked the induction of FOXP3 mRNA expression to the levels demonstrated in the AS or CV supernatants (Fig. 3C). Control Ab did not impact the COX-2 S tumor supernatant-mediated induction of FOXP3 mRNA expression (Fig. 3C).

Table I. CD25 expression in CD4$^+$CD25$^{dim}$ and CD4$^+$CD25$^{bright}$ following magnetic bead purification

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<tr>
<th>CD4 $^+$CD25$^{dim}$</th>
<th>CD4 $^+$CD25$^{bright}$</th>
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<tr>
<td>% Positive (MFI)</td>
<td>% Positive (MFI)</td>
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<tr>
<td>24.2 ± 19</td>
<td>94 ± 2.7</td>
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*CD25 surface expression and PGE$_2$-mediated modulation of CD25 in CD4$^+$ PBL. Data summarize (n = 8) the CD25 expression of CD4$^+$CD25$^{dim}$ and CD4$^+$CD25$^{bright}$ T cell populations following cell purification (Miltenyi Treg Cell Isolation kit). Results are expressed as percentage of CD25 expression within the CD4$^+$ population. Values in parentheses indicate the mean fluorescence intensity (MFI) of CD25 expression based on density plot analysis.

FIGURE 2. PGE$_2$ down-regulates CD25 expression in CD3-activated CD4$^+$ lymphocytes. A, PGE$_2$-treated, CD3-activated CD4$^+$CD25$^{dim}$ T cells up-regulate CD25 expression. Double-positive CD4$^+$CD25$^+$ cells were measured in unstimulated (top panel) and anti-CD3-stimulated (bottom panel), PGE$_2$-treated CD4$^+$CD25$^+$ T cells. Density plots of a representative experiment of four performed are shown. A, The number inserted in the upper right quadrant indicates the percentage of double-positive cells (CD4$^+$CD25$^+$). B, PGE$_2$ decreased CD25 expression in both CD4$^+$CD25$^{dim}$ (B) and CD4$^+$CD25$^{bright}$ starting populations (C). CD25 was measured in anti-CD3-stimulated, CD4$^+$CD25$^{dim}$ T cells and in CD4$^+$CD25$^{bright}$ T reg cells without (lower panel) or with (+) PGE$_2$ (26 M; bottom panel) at the indicated time points. Results were obtained from a donor different from that in A. In the histograms, dotted lines represent the isotype control, and bold lines indicate surface marker staining. B, The numbers inserted indicate the mean fluorescent intensity (MFI) of CD25 expression. Results are representative of one experiment of three performed.
PGE₂ up-regulates FOXP3 mRNA and protein expression and induces FOXP3 promoter activity

To determine the mechanisms underlying PGE₂-dependent regulation of FOXP3, we analyzed the expression of FOXP3 mRNA and protein levels in untreated or PGE₂-treated (13 and 26 μM) Jurkat T cells in the presence or the absence of TCR-activating stimuli. We found that PGE₂ up-regulated FOXP3 mRNA levels in both TCR-activated and non-activated Jurkat T cells (Fig. 4, A and C). Consistent with our findings in PBL, PGE₂ induced the greatest increase in FOXP3 in TCR-stimulated Jurkat T cells. Western blot analysis showed a similar pattern of expression (Fig. 4B). Non-activated Jurkat T cells, in fact, expressed FOXP3 protein that was significantly up-regulated by both PGE₂ concentrations tested (13 and 26 μM). However, maximum FOXP3 up-regulation was obtained in Jurkat T cells stimulated with PGE₂ (26 μM) and activated with PMA plus ionomycin (Fig. 4B). Finally, in promoter reporter assays, Jurkat T cells transfected with a FOXP3 promoter construct (FOXP3-SubD) demonstrated significantly enhanced FOXP3 promoter activity in response to PGE₂ and costimulatory signals (Fig. 4C). This suggests that PGE₂ operates to induce FOXP3 at the level of mRNA transcription.

**Discussion**

Several in vitro studies have reported an immunosuppressive role of PGE₂ in T cell proliferation. Most studies focused on the direct effect of PGE₂ on the responding T cell in which elevated levels of intracellular cAMP were associated with reduced IL-2 production (36, 37). Several other pathways of PGE₂-induced immunosuppression have been described, including inhibition of polyamine synthesis, inhibition of intracellular calcium release, and suppression of p59 (Fyn) protein tyrosine kinase activity (38). However, the contribution of PGE₂ in modulating T regulatory cell activity has not previously been defined.

A specific subset of T reg cells bearing a CD4⁺CD25⁺ T cell phenotype has now been the focus of extensive investigation (1–5, 35). These T cells, endowed with distinct immunomodulatory properties, are acknowledged as fundamental in the prevention of autoimmune and may be important in preventing effective immune responses in malignancy (17–22). Although naturally occurring CD4⁺CD25⁺ T reg cells develop directly from CD4⁺ precursors in the thymus, a body of work has now demonstrated the induction of cells with regulatory function in the periphery from naïve CD4⁺ T cells in response to a variety of stimuli (6–12).

In this study we report a novel mechanism of PGE₂-induced immunosuppression that occurs through the modulation/induction of human T reg cell function. Our results show that PGE₂ not only enhances the suppressive capacity of in vitro purified CD4⁺CD25⁺ T reg cells, but also induces a CD4⁺CD25⁺ T cell population to develop T reg cell function. Analysis of this newly induced T reg cell-like population revealed that PGE₂ exposure conferred acquisition of FOXP3 expression consistent with the phenotypic feature seen in naturally occurring T reg cells. The original CD25⁺ population became CD25bright upon anti-CD3 activation. However, PGE₂ exposure markedly down-regulated the surface expression of CD25 in these CD3-stimulated CD4⁺CD25⁺ T cells that typically developed CD25bright expression upon stimulation. The down-regulation of CD25, the α-chain of the high affinity IL-2R, was also evident in the purified CD4⁺CD25⁺ T reg cells exposed to the same concentration of PGE₂. Constitutive expression of CD25 commonly has been used to define CD4⁺CD25⁺ T reg cells and IL-2 has been implicated as an important modulator of T reg activity, in vivo (39, 40).

**Table II.** CD25 expression in CD4⁺CD25dim T cells treated with or without PGE₂

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Percentage positive and MFI

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*CD25 surface expression and PGE₂-mediated modulation of CD25 in CD4⁺ PBL. Data summarize (n = 4) the analysis of CD25 expression in CD4⁺CD25dim T reg cells in a time-course flow-cytometry experiment from PBL donors (n = 4), as described in Materials and Methods. Briefly, magnetic bead-purified CD4⁺CD25dim, pretreated with or without PGE₂, were incubated with anti-CD3 plate-bound Ab at the indicated time points. Surface CD25 expression was then measured by flow cytometry. Results are expressed as percentage of CD25 expression within the CD4⁺ population. Values in parentheses indicate the mean fluorescence intensity (MFI) of CD25 expression based on density plot analysis.

**Table III.** CD25 expression in CD4⁺CD25bright T reg cells treated with or without PGE₂

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*CD25 surface expression and PGE₂-mediated modulation of CD25 in CD4⁺ PBL. Data summarize (n = 4) the analysis of CD25 expression in CD4⁺CD25bright T reg cells in a time-course flow-cytometry experiment from PBL donors (n = 4), as described in Materials and Methods. Briefly, magnetic bead-purified CD4⁺CD25bright, pretreated with or without PGE₂, were incubated with anti-CD3 plate-bound Ab at the indicated time points. Surface CD25 expression was then measured by flow cytometry. Results are expressed as percentage of CD25 expression within the CD4⁺ population. Values in parentheses indicate the mean fluorescence intensity (MFI) of CD25 expression based on density plot analysis.
finding of PGE₂-mediated down-regulation of IL-2Rα is consistent with previous studies showing decreased IL-2 production and IL-2Rα expression in TCR-activated, PGE₂-stimulated T cells (37, 38, 42, 43). Thus, the PGE₂-mediated down-regulation of IL-2Rα may limit typical autocrine and paracrine IL-2-dependent function, because Ab-blocking studies could not reverse the inhibitory function of both PGE₂-treated CD4⁺CD25⁺ and CD4⁺CD25⁻ T reg cells. Alternatively, IL-2 and IL-7 may activate different signal transduction pathways, ultimately leading to different outcomes (43–46). Additional studies will be required to define the differences in response to IL-2 and IL-7.

PBL-derived T reg cells have been categorized into T regulatory 1 or Th3 based on their pattern of cytokine secretion, because they have been shown to preferentially produce IL-10 or TGF-β, respectively (46). However, there is as yet no clear consensus regarding their phenotype or their ontogeny (5, 46). Our findings indicate that compared with untreated CD4⁺CD25⁺ T cells, PGE₂-induced T reg-like cells secrete lower levels of IL-10 and similar levels of TGF-β (data not shown). Neither of these cytokines appeared to significantly contribute to the inhibitory effect of either PGE₂-treated CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells (data not shown). Taken together, our data indicate that PGE₂-induced CD4⁺CD25⁺ T cells share common features of both thymic and peripheral T reg cells (4, 5, 47, 48).

The transcription factor FOXP3 exerts a key role in the development and function of T reg cells (1–5, 46). This separates FOXP3 from other T reg cell-associated markers such as CD25, CD25/FOXP3, FOXP3, and CD25/FOXP3. These cell subsets represented the T reg cell phenotype and function as a whole (46). Our findings suggest that PGE₂-induced T reg cells are characterized by a unique combination of FOXP3 expression and proliferation. PGE₂-induced FOXP3 expression did not affect the down-regulation of IL-2Rα. This finding is consistent with previous studies showing decreased IL-2 production and IL-2Rα expression in TCR-activated, PGE₂-stimulated T cells (37, 38, 42, 43). Thus, the PGE₂-mediated down-regulation of IL-2Rα may limit typical autocrine and paracrine IL-2-dependent function, because Ab-blocking studies could not reverse the inhibitory function of both PGE₂-treated CD4⁺CD25⁺ and CD4⁺CD25⁻ T reg cells. Alternatively, IL-2 and IL-7 may activate different signal transduction pathways, ultimately leading to different outcomes (43–46). Additional studies will be required to define the differences in response to IL-2 and IL-7.
glucocorticoid-induced TNFR family-related protein, and 
CTLA-4, which may be more generally expressed by activated T 
cells (35, 36). In our study, PGE2-dependent T reg differentiation 
was indeed associated with induction of the transcription factor 
FOXP3, whose expression was also significantly up-regulated by 
PGE2 in purified CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^-\) T cells. Consis-
tent with previous reports in which TGF-\(\beta\) was investigated (6 – 8), 
PGE2-mediated T reg cell differentiation and 
FOXP3 induction 
were evident predominantly in the presence of costimulation by 
either anti-CD3 or allogeneic dendritic cells (data not shown). Us-
ing a variety of approaches, several studies are now modeling the 
differentiation of cells with T regulatory function arising in the 
periphery (6 –12, 48). For example, recent studies in mice and 
humans, have demonstrated that CD4\(^+\)CD25\(^-\) T cells treated with 
TGF-\(\beta\) in the presence of appropriate costimulation lead to T reg 
cell differentiation with the capacity for suppressive function in 
vitro (6, 7). It is unclear whether these extrathymic T reg cells 
represent altered states of differentiation or belong to a unique T 
cell lineage (48).

Our findings suggest a role for PGE2 in modulating T reg cell 
function and differentiation. In conditions of PGE2 overproduction, 
such as lung cancer that have previously been demonstrated to 
have high COX-2 expression and PGE2 production (16, 49) have 
also been noted to have increased T reg cell number and function 
within the PBL as well as the tumor-infiltrating lymphocyte pop-
ulations (17–22). Extending these previous reports, we found that 
NSCLC cell lines overexpressing COX-2 were able to induce 
FOX3 gene expression in CD4\(^+\)CD25\(^-\) T cells in a PGE2-de-
pendent manner. We have recently reported that these relationships 
are operative in vivo in murine lung cancer models (50). Thus, 
taken together, our data indicate that regulation of PGE2 produc-
tion may, in turn, modulate T reg cell development and function. 
These findings suggest new therapeutic strategies targeting COX-
2/PGE2 in the prevention and treatment of cancer. This could in-
clude addition of COX-2 inhibitor treatment to immune-based 
therapies.

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Disclosures
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FIGURE 4. PGE2 enhances FOXP3 mRNA expression, gene transcription, and 
protein. A, Jurkat T cells were activated for 18 h as indicated. FOXP3 
mRNA expression was quantified by real-time PCR as described in Materials and Methods. B, Top panel, FOXP3 protein was analyzed by Western blot 
after stimulation for 18 h as indicated. B, Bottom panel, Densitometric analysis of FOXP3 expression of the Western blot. One representative result of two 
independent experiments performed is shown. C, PGE2 induces FOXP3 promoter activity. Jurkat T cells cotransfected with FOXP3 promoter and firefly 
luciferase reporter gene constructs (FOX3-SubD) or with Renilla luciferase gene (CV) were stimulated for 18 h as indicated. FOXP3 promoter expression 
was analyzed by a dual luciferase reporter assay system, and relative luciferase activity was expressed as the ratio of firefly to Renilla luciferase. A 
representative experiment of three performed is shown.
References


