Prostaglandin E₂ Induces FOXP3 Gene Expression and 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

*J Immunol* 2005; 175:1483-1490; doi: 10.4049/jimmunol.175.3.1483
http://www.jimmunol.org/content/175/3/1483

**Why The JI?**
- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*(average)*

**References** This article cites 49 articles, 30 of which you can access for free at: http://www.jimmunol.org/content/175/3/1483.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2005 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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.

Materials and Methods

Cell isolation and culture

Human CD4+CD25+ T reg cells and CD4+CD25− T cells were purified from enriched buffy coat from healthy volunteers using the T reg cell isolation kit (Miltenyi Biotec) and the AutoMACS cell sorter (Miltenyi Biotec). An institutional review board approval was obtained, and all donors signed informed consent. Cell purity assessed by flow cytometry was ≥92%. Before being used in different experimental conditions, purified CD4+CD25+ and CD4+CD25− T cell fractions were preincubated for 24 h in X-Vivo 15 (BioWhittaker), 10% FBS, and 1% human serum AB (Gemini) (24) with or without 16,16-dimethyl-PGE2 (Cayman Chemical; 13 or 26 μM). In some experiments, CD4+CD25− T cells (2 × 10^5) were preincubated for 18–24 h with 2 ml of undiluted tumor supernatant obtained from H157 (human squamous cell carcinoma; American Type Culture Collection) NSCLC, genetically modified to express COX-2 sense (S), COX-2 antisense (AS), or control empty vector (CV) (25). A 2.0-kb cDNA fragment of human COX-2 (provided by Dr. H. Herschman, University of California, Los Angeles, CA) was cloned in S and AS orientations in the retroviral vector pLNCX (BD Clontech) as previously described (25). For each cell line, an ~10-fold higher level of COX-2 expression and PGE2 production was noted in COX-2 S compared with parental controls or CV (25). To neutralize the activity of the PGE2 contained in the COX-2 S supernatant, anti-PGE2 (10 μg/ml) was added.
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 isotype (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 T cells were reactivated 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 μg/ml; Sigma-Aldrich) and ionomycin (1 μg/ml)-coated, 96-well plates (BD Biosciences) were used in the proliferation assays (2 ng/ml equivalent to 4 U/ml and 20 IU/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-2 (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′-CAGATGAGATGTCGGTCCACCA-3′; reverse, 5′-CTTCTCGGCACTGTCCTTT-3′. FOXP3 primers were synthesized by Integrated DNA Technologies. For β-actin, primers were: forward, 5′-GATGAGATGTCGGTCCACCA-3′; and reverse, 5′-CACCTTACCCGATCACGTTT-3′.

REGULATORY EFFECT OF PGE2 IN HUMAN CD4+ T CELLS

FOXP3 promoter cotransfection of Jurkat T cells and dual luciferase assay
A 350-bp fragment (FOXP3-subD) containing the FOXP3/scurfin gene intron 0 upstream from the initiation codon, located within the 65,825–65,475 bp region of human chromosome X, multiple clone map pH.23, was inserted into a firefly luciferase reporter vector (pGL3-basic; Promega) as previously described (28, FOXP3-subD 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 (BacTec Systems; 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 × 10⁶ cells). The protein concentration in the cell lysate was determined using a bichinchoninic 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 Immunolabel). 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 PGE2 (26 μM)). Addition of IL-7 (2 ng) partially overcame the PGE2-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 PGE2 (26 μM)), 0.89 ± 0.07 (CD4+CD25−), and 0.69 ± 0.22 (CD4+CD25− plus PGE2 (26 μM)). In contrast, IL-2 (20 IU/ml) did not overcome the PGE2-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 PGE2 (26 μM)), 0.64 ± 0.36 (CD4+CD25−), and 0.31 ± 0.16 (CD4+CD25− plus PGE2 (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 PGE2-treated stimulator cells (CD4+CD25− or CD4+CD25+), both PGE2-treated populations maintained the capacity to inhibit T cell proliferation (Fig. 1E). These data taken together suggest that the PGE2-induced T regulatory function does not require cell contact. We have previously found that PGE2 is a potent inducer of lymphocyte IL-10 gene transcription (15), and PGE2 has also been reported to increase TGF-β expression (33–35). However, neither anti-IL-10 nor anti-TGF-β neutralizing Abs were able to reverse the PGE2-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-β to PGE2-induced cell-contact independent T regulatory cell function.
Down-regulation of CD25 expression in PGE₂-treated CD4⁺ T cells

Because T reg cells are identified as a CD4⁺CD25bright T cell subset (36), we analyzed whether the PGE₂-treated CD4⁺CD25dim starting population acquired the same phenotype of naturally occurring, thymic-derived CD4⁺CD25bright T reg cells. As shown in Table I, CD25 was differentially expressed in magnetic bead-purified CD4⁺CD25dim and CD4⁺CD25bright T cells. Indeed, with (Fig. 2A and Table II) or without PGE₂ preincubation (data not shown), CD4⁺CD25dim 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 PGE₂, CD25 expression declined in both CD4⁺CD25dim T cells (Fig. 2B, bottom left, and Table II) and CD4⁺CD25bright T reg cells (Fig. 2B, bottom right, and Table III), compared with their counterparts without PGE₂ 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-β1, lymphocyte activation Ag 3, PD-1, CD103, CD122, and CCR4, were either not altered or were down-regulated by PGE₂ (data not shown).

PGE₂ 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 PGE₂ 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 PGE₂ (26 μM) 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 PGE₂ (26 μM), FOXP3 mRNA expression was also significantly induced, consistent with the acquisition of a T reg cell suppressive function (Fig. 3B). Thus, PGE₂ 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 PGE₂ (17–22). Thus, to determine the importance of tumor-derived

---

Table I. CD25 expression in CD4⁺CD25dim and CD4⁺CD25bright following magnetic bead purification

<table>
<thead>
<tr>
<th>CD4⁺CD25dim</th>
<th>CD4⁺CD25bright</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Positive (MFI)</td>
<td>% Positive (MFI)</td>
</tr>
<tr>
<td>24.2 ± 19 (51 ± 7.7)</td>
<td>94 ± 2.7 (247 ± 113.8)</td>
</tr>
</tbody>
</table>

*CD25 surface expression and PGE₂-mediated modulation of CD25 in CD4⁺ PBL. Data summarize (n = 8) the CD25 expression of CD4⁺CD25dim and CD4⁺CD25bright 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. PGE₂ down-regulates CD25 expression in CD3-activated CD4⁺ lymphocytes. A, PGE₂-treated, CD3-activated CD4⁺CD25dim T cells up-regulate CD25 expression. Double-positive CD4⁺CD25⁺ cells were measured in unstimulated (top panel) and anti-CD3-stimulated (bottom panel), PGE₂-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⁺). PGE₂ decreased CD25 expression in both CD4⁺CD25dim (B) and CD4⁺CD25bright starting populations (C). CD25 was measured in anti-CD3-stimulated, CD4⁺CD25dim T cells and in CD4⁺CD25bright T reg cells without (− top panel) or with (+) PGE₂ (26 μM; 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 fluorescence intensity (MFI) of CD25 expression. Results are representative of one experiment of three performed.

---

PGE₂, we used COX-2 gene-modified NSCLC cells as an experimental model for the tumor site, which is often enriched for PGE₂. 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-PGE₂ 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).
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 autoimmunity 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). Our

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

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>CD4CD25dim</th>
<th>CD4CD25dim + PGE₂</th>
<th>CD4CD25dim</th>
<th>CD4CD25dim + PGE₂</th>
<th>CD4CD25dim</th>
<th>CD4CD25dim + PGE₂</th>
<th>CD4CD25dim</th>
<th>CD4CD25dim + PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>64 (277)</td>
<td>40 (71)</td>
<td>74 (833)</td>
<td>47 (186)</td>
<td>91 (1516)</td>
<td>59 (559)</td>
<td>95 (1091)</td>
<td>82 (614)</td>
</tr>
<tr>
<td>II</td>
<td>59 (125)</td>
<td>41 (42)</td>
<td>74 (272)</td>
<td>39 (64)</td>
<td>94 (934)</td>
<td>64 (364)</td>
<td>96 (1039)</td>
<td>74 (473)</td>
</tr>
<tr>
<td>III</td>
<td>75 (101)</td>
<td>55 (36)</td>
<td>63 (102)</td>
<td>25 (21)</td>
<td>71 (719)</td>
<td>47 (102)</td>
<td>96 (1055)</td>
<td>78 (638)</td>
</tr>
<tr>
<td>IV</td>
<td>69 (144)</td>
<td>46 (66)</td>
<td>74 (668)</td>
<td>50 (282)</td>
<td>89 (1599)</td>
<td>63 (698)</td>
<td>96 (1360)</td>
<td>65 (676)</td>
</tr>
</tbody>
</table>

Percentage positive and MFI

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 Treg cells treated with or without PGE₂

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>CD4CD25bright</th>
<th>CD4CD25bright + PGE₂</th>
<th>CD4CD25bright</th>
<th>CD4CD25bright + PGE₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>87 (179)</td>
<td>70 (60)</td>
<td>79 (256)</td>
<td>69 (52)</td>
</tr>
<tr>
<td>II</td>
<td>89 (78)</td>
<td>79 (41)</td>
<td>84 (101)</td>
<td>47 (37)</td>
</tr>
<tr>
<td>III</td>
<td>94 (66)</td>
<td>84 (44)</td>
<td>97 (76)</td>
<td>28 (45)</td>
</tr>
<tr>
<td>IV</td>
<td>81 (45)</td>
<td>68 (39)</td>
<td>87 (56)</td>
<td>74 (28)</td>
</tr>
</tbody>
</table>

Percentage positive and MFI

Downloaded from http://www.jimmunol.org/ by guest on December 11, 2017
finding of PGE$_2$-mediated down-regulation of IL-2R$\alpha$ is consistent with previous studies showing decreased IL-2 production and IL-2R$\alpha$ expression in TCR-activated, PGE$_2$-treated T cells (37, 38, 42, 43). Thus, the PGE$_2$-mediated down-regulation of IL-2R$\alpha$ may limit typical autocrine and paracrine IL-2-dependent function, because Ab-blocking studies could not reverse the inhibitory function of both PGE$_2$-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.

Figure 3. PGE$_2$ induces FOXP3 mRNA expression in both CD4$^+$CD25$^+$ T reg cells and CD4$^+$CD25$^-$ T cells. Purified CD4$^+$CD25$^+$ T reg cells (A) and CD4$^+$CD25$^-$ T cells (B) were incubated without (−) or with (+) PGE$_2$ (26 μM) for 24 h. C, Alternatively, CD4$^+$CD25$^-$ T cells were cultured for 24 h with medium alone or in tumor supernatant from COX-2 S (with or without anti-PGE$_2$ Ab (10 μg/ml) or mouse IgG control Ab (10 μg/ml)), COX-2 AS, or CV-transfected H157. T cell proliferation was quantified after 48-h activation with plate-bound anti-CD3 Ab by real-time PCR as described in Materials and Methods. In CD4$^+$CD25$^+$ T reg cells treated with or without PGE$_2$ (A), the fold increase in FOXP3 mRNA expression is relative to FOXP3 mRNA expression in CD4$^+$CD25$^-$ cells. In PGE$_2$-treated CD4$^+$CD25$^-$ cells (B), the fold change in FOXP3 mRNA relative to that in untreated CD4$^+$CD25$^-$ cells. In COX-2 S T cells cultured with gene-modified H157 supernatant (C), the fold change in FOXP3 was relative to that in CD4$^+$CD25$^-$ cells in medium alone. Results are expressed as the mean (±SE) of one experiment of at least three performed in triplicate from different donors. Asterisks indicate statistical significance compared with the control value.

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,
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. Consistent with previous reports in which TGF-β 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). Using 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-β 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 described in several malignancies (17–22), increased T reg cell differentiation and function could contribute to tumor-induced immunosuppression. Consistent with this concept, malignancies 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 populations (17–22). Extending these previous reports, we found that NSCLC cell lines overexpressing COX-2 were able to induce FOXP3 gene expression in CD4+CD25− T cells in a PGE2-dependent 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 production 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 include addition of COX-2 inhibitor treatment to immune-based therapies.

Acknowledgments
We thank Lauren Winter and Sandra Tran for their technical assistance. Flow cytometry was performed in the Flow Cytometry Core Facility at the University of California-Los Angeles Jonsson Comprehensive Cancer Center and Center for AIDS Research.

Disclosures
The authors have no financial conflict of interest.
References


