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Prostaglandin E$_2$ Induces *FOXP3* Gene Expression and T Regulatory Cell Function in Human CD4$^+$ T Cells[1]

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[2]

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 PGE$_2$ enhances the in vitro inhibitory function of human purified CD4$^+$CD25$^+$ T reg cells. Moreover, PGE$_2$ induces a regulatory phenotype in CD4$^+$CD25$^-$ T cells. PGE$_2$-treated T cell-mediated inhibition of anti-CD3-stimulated lymphocyte proliferation did not require cell contact. Phenotypic analysis revealed that PGE$_2$ diminished CD25 expression in both CD4$^+$CD25^{dim} T cells and CD4$^+$CD25^{bright} T reg cells. PGE$_2$ 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 PGE$_2$ significantly induced *FOXP3* in CD4$^+$CD25$^-$ T cells. Finally, PGE$_2$ up-regulated *FOXP3* at both mRNA and protein levels and enhanced *FOXP3* promoter activity. This is the first report indicating that PGE$_2$ can modulate *FOXP3* expression and T reg function in human lymphocytes. *The Journal of Immunology,* **2005, 175: 1483–1490.**

Naturally occurring regulatory T (T reg)[3] cells have been identified as CD4$^+$CD25$^+$ T lymphocytes that specifically express the forkhead transcription factor forkhead/winged helix transcription factor gene (*FOXP3*) (1–3). Their importance resides in their pivotal role in the maintenance of immunological tolerance (4). Although the suppressive capacity of T reg cells has been very well demonstrated, the mechanisms controlling their thymic origin and potential peripheral development have not been fully defined (5). Recent studies indicate that T cells with regulatory function may arise in the periphery upon conversion of CD4$^+$CD25$^-$ T cells into CD4$^+$CD25$^+$ T reg cells through *FOXP3* gene induction in response to a variety of stimuli (6–12).

PGE$_2$ has diverse effects on the regulation and activity of CD4$^+$ T cells, including modulation of proliferation (13). Elevated tumor cyclooxygenase-2 (COX-2) and PGE$_2$ levels have been described in several malignancies, where they have been implicated in suppression of antitumor immunity (15–16). In particular, the CD4$^+$CD25$^+$ T reg cell population has been found to be significantly increased in PBL and within the tumor-infiltrating lymphocytes of epithelial cancers, including non-small cell lung cancer (NSCLC) (17–23). Given that COX-2 overexpression and PGE$_2$ overproduction are highly associated with the progression of this malignancy (16), we hypothesized that PGE$_2$ could contribute to the tumor-induced immunosuppression through modulation of T reg cell function. In this study we demonstrate that PGE$_2$ enhances the inhibitory capacity of in vitro purified human CD4$^+$CD25$^+$ T reg cells and induces a suppressive regulatory phenotype in CD4$^+$CD25$^-$ T cells. The PGE$_2$-dependent acquisition of T reg cell function was correlated with induction of FOXP3 gene and protein expression. The enhanced inhibitory activity of PGE$_2$-treated CD4$^+$CD25$^+$ T reg cells was also associated with significant up-regulation of *FOXP3*. This report highlights novel roles for PGE$_2$ in controlling the generation and function of T reg cells.

Materials and Methods

Cell isolation and culture

Human CD4$^+$C25$^+$ 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-PGE$_2$ (Cayman Chemical; 13 or 26 μM). In some experiments, CD4$^+$CD25$^-$ T cells (2 × 10^6) 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 PGE$_2$ production was noted in COX-2 S compared with parental controls or CV (25). To neutralize the activity of the PGE$_2$ contained in the COX-2 S supernatant, anti-PGE$_2$ (10 μg/ml; provided by J. Portanova, Searle, St.

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$^+$C25$^+$ 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 PGE$_2$ and cultured with or without anti-CD3 stimulation, were resuspended in PBS/2% FBS and labeled with human anti-CD25 PE (Miltenyi Biotec) and human anti-CD4 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$^+$C25$^+$ T reg cells were pretreated with PGE$_2$ (13 or 26 μM) for 24 h. PGE$_2$ was removed by washing before starting the proliferation assay. To rule out the possibility that residual PGE$_2$ would be transferred to the second culture, we performed preliminary experiments in which T cells were pretreated with PGE$_2$ (26 μM; Cayman Chemical; catalogue no. 14010) for 12–18 h. The presence of PGE$_2$ in the supernatant was then assessed by a specific enzyme immunoassay (PGE$_2$ EIA kit; Cayman Chemical) after cell washing. The latter procedure was determined to be effective in removing >99% of the added PGE$_2$ (data not shown). Both CD4$^+$CD25$^-$ and CD4$^+$C25$^+$ T cell populations, with or without PGE$_2$ pretreatment, were then cultured at a 1:1 ratio with autologous CD4$^+$CD25$^-$ responder T cells (1 × 10^5) in anti-CD3 plate-bound, 96-well plates. In some experiments IL-2 (Proleukin; biological activity, 18 × 10^6 IU/1.1 mg; Chiron) or IL-7 (biological activity, 2 × 10^6 U/mg; 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 × 10^5 untreated or PGE$_2$-treated CD4$^+$CD25$^-$ T cells and untreated or PGE$_2$-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 × 10^5) 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 GTT CCA CAA CAT GCG AC-3′; and reverse, 5′-ATT GAG TGT CCG CTG CTT CT-3′. FOXP3 primers were synthesized by Integrated DNA Technologies. For β-actin, primers were: forward, 5′-GATGAGATTGGCATGGCTTT-3′; and reverse, 5′-CACCTTCACCGTTCCAGTTT-3′.

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 pII.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). PGE$_2$ (13 μM), PMA (20 ng/ml) plus ionomycin (1 μg/ml) (PI), and PMA, ionomycin, and PGE$_2$ 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 (Bacterial 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 × 10^5 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 Immunochemical). 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

PGE$_2$ 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 PGE$_2$ in mediating suppression of lymphocyte proliferation, highlighting the role of calcium-dependent mechanisms (29, 30). In the current study we first investigated whether exogenous PGE$_2$ 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 PGE$_2$ for 24 h in vitro (Fig. 1A). Treatment of CD4$^+$CD25$^+$ T reg cells with escalating doses of PGE$_2$ (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$^+$ + PGE$_2$; 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 PGE$_2$ displayed numerous homogeneously distributed cell clusters, indicative of ongoing proliferation. In contrast, wells containing stimulator T cells pretreated with PGE$_2$ 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 PGE$_2$ (13 or 26 μM) conferred regulatory T cell function (Fig. 1B). In fact, the PGE$_2$-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 PGE$_2$ were similar to those induced by untreated CD4$^+$CD25$^+$T

FIGURE 1. *A*, PGE$_2$ enhances T reg cell function. Purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells, precultured with or without PGE$_2$, were mixed with autologous CD4$^+$ cells in anti-CD3-bound, 96-well plates for 5 days. *B*, PGE$_2$ induces T reg cell activity in CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were precultured with increasing concentrations of PGE$_2$ before a 5-day proliferation assay. *C* and *D*, IL-7, but not IL-2, overcomes suppression of T cell proliferation. IL-7 (*C*) or IL-2 (*D*) was added on day 1 to the proliferation assay described above. *E*, PGE$_2$ mediates T reg cell activity without the requirement for cell contact. Both untreated and PGE$_2$-treated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells were separated from dendritic cell-activated CD4$^+$CD25$^-$ T cells by Transwell inserts. In all experiments, cell proliferation was measured by BrdU incorporation. Results are expressed as the mean ($\pm$SD) of one representative experiment of at least three performed in triplicate from different donors.
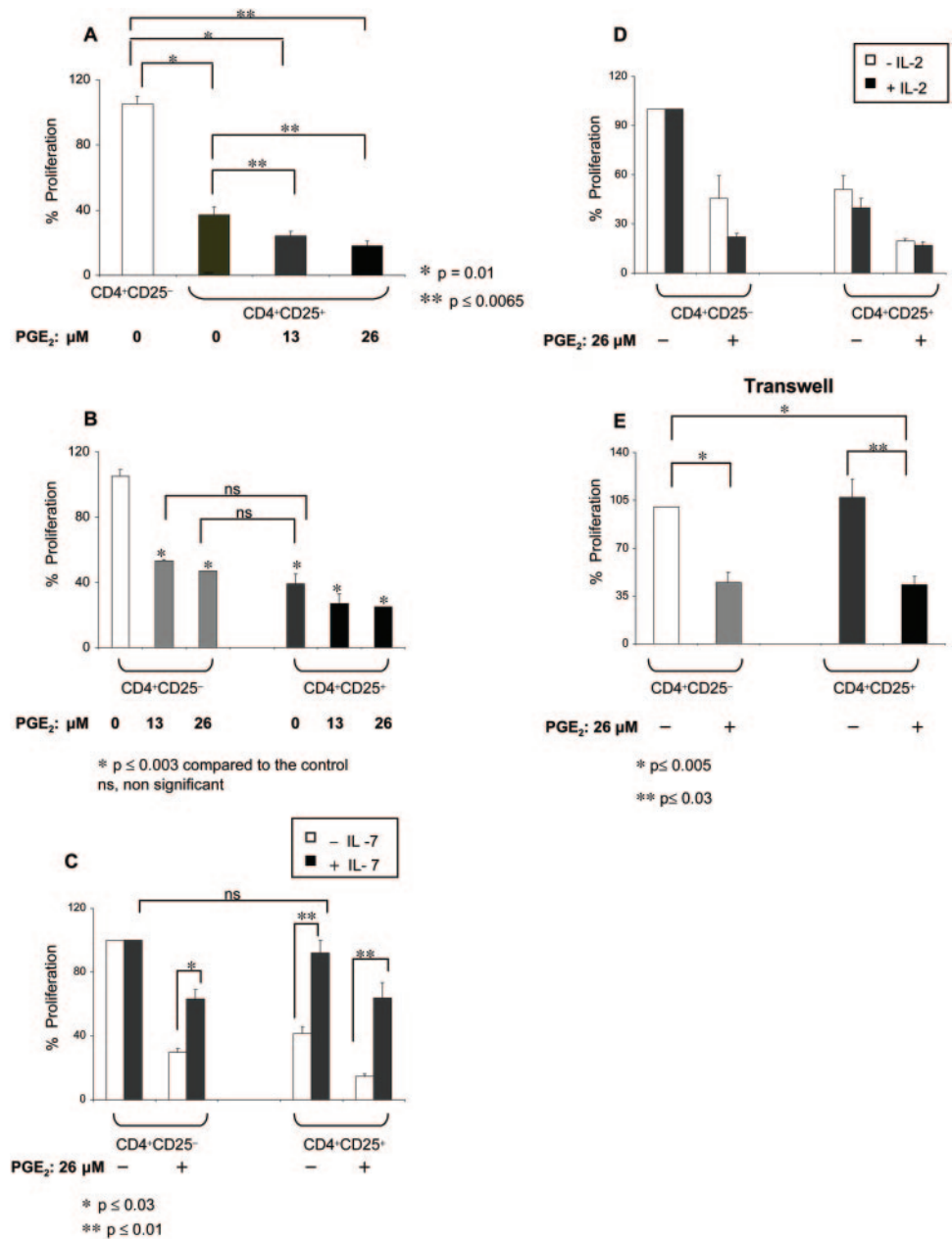
reg cells (Fig. 1*B*). The T cell proliferations, expressed as OD values of BrdU incorporation, obtained from three different donors were 1.26 ± 0.39 (CD4$^+$CD25$^-$) and 0.65 ± 0.40 (CD4$^+$CD25$^-$ plus PGE$_2$ (26 μM)). Addition of IL-7 (2 ng) partially overcame the PGE$_2$-mediated suppression and reversed the CD4$^+$CD25$^+$ T reg cell-mediated inhibition of proliferation (Fig. 1*C*). 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. 1*D*). 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 ($\geq$2 ng/ml) and a high concentration of IL-2 ($\geq$100 IU/ml) demonstrated the capacity to induce proliferation of T reg cells (data not shown) in agreement with previous reports (31, 32). Interestingly, when CD4$^+$CD25$^-$ 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. 1*E*). 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-β expression (33–35). However, neither anti-IL-10 nor anti-TGF-β 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-β to PGE$_2$-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⁺CD25^bright T cell subset (36), we analyzed whether the PGE₂-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. 2*A* and Table II) or without PGE₂ preincubation (data not shown), CD4⁺CD25^dim up-regulated CD25 as early as 24 h after stimulation with anti-CD3 plate-bound Ab (Fig. 2*A*). These T cells maintained high levels of CD25 expression throughout a 96-h culture period (Fig. 2*A*). However, when anti-CD3 Ab-activated T cells were pretreated with PGE₂, CD25 expression declined in both CD4⁺CD25^dim T cells (Fig. 2*B*, *bottom left*, and Table II) and CD4⁺CD25^bright T reg cells (Fig. 2*B*, *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. 3*A*). Furthermore, a 24-h preincubation with PGE₂ (26 μM) resulted in >4-fold up-regulation of *FOXP3* (Fig. 3*A*). 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. 3*B*). 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

FIGURE 2. PGE₂ down-regulates CD25 expression in CD3-activated CD4⁺ lymphocytes. *A*, PGE₂-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*), 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⁺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 (−; *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 fluorescent intensity (MFI) of CD25 expression. Results are representative of one experiment of three performed.

Table I. *CD25 expression in CD4⁺CD25^dim and CD4⁺CD25^bright following magnetic bead purification^a*

	CD4⁺CD25^dim		CD4⁺CD25^bright	
	% Positive	(MFI)	% Positive	(MFI)
	24.2 ± 19	(51 ± 7.7)	94 ± 2.7	(247 ± 113.8)

^a CD25 surface expression and PGE₂-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.
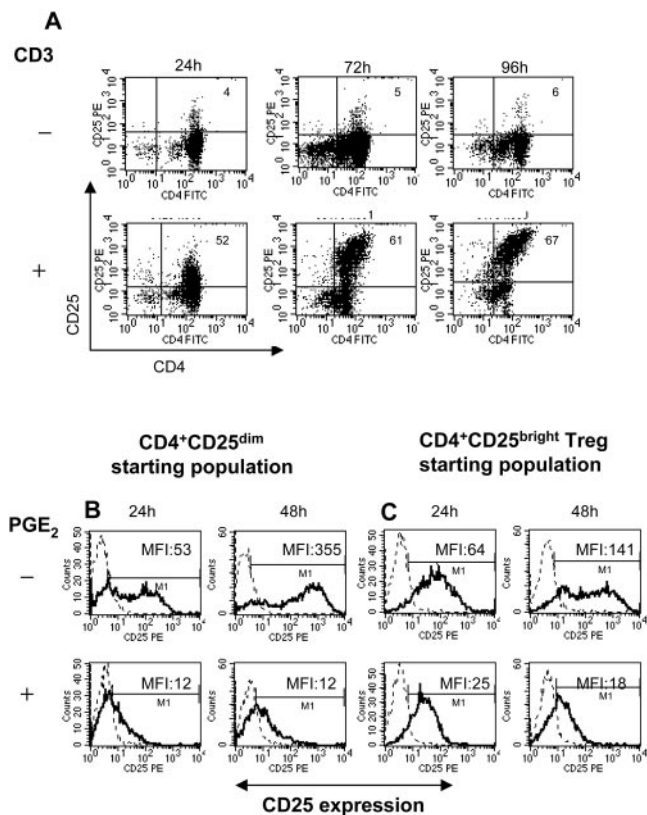
PGE₂, we used COX-2 gene-modified NSCLC cells as an experimental model for the tumor site, which is often enriched for PGE₂. For this purpose we genetically modified NSCLC cell lines to express COX-2 S, COX-2 AS, or empty CV, as described in *Materials and Methods* and as we have previously reported (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. 3*C*). Control Ab did not impact the COX-2 S tumor supernatant-mediated induction of *FOXP3* mRNA expression (Fig. 3*C*).

Table II. *CD25 expression in CD4$^+$CD25^{dim} T cells treated with or without PGE$_2$[a]*

	24 h		48 h		72 h		96 h	
Sample No.	CD4CD25^{dim}	CD4CD25^{dim} + PGE$_2$	CD4CD25^{dim}	CD4CD25^{dim} + PGE$_2$	CD4CD25^{dim}	CD4CD25^{dim} + PGE$_2$	CD4CD25^{dim}	CD4CD25^{dim} + PGE$_2$
				Percentage positive and MFI				
I	64 (277)	40 (71)	74 (833)	47 (186)	91 (1516)	59 (559)	95 (1091)	82 (614)
II	59 (125)	41 (42)	74 (272)	39 (64)	94 (934)	64 (364)	96 (1039)	74 (473)
III	75 (101)	55 (36)	63 (102)	25 (21)	71 (719)	47 (102)	96 (1055)	78 (638)
IV	69 (144)	46 (66)	74 (668)	50 (282)	89 (1599)	63 (698)	96 (1360)	65 (676)

[a] CD25 surface expression and PGE$_2$-mediated modulation of CD25 in CD4$^+$ PBL. Data summarize ($n = 4$) the analysis of CD25 expression in CD4$^+$CD25^{dim} 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$^+$CD25^{dim}, pretreated with or without PGE$_2$ 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.

PGE$_2$ up-regulates FOXP3 mRNA and protein expression and induces FOXP3 promoter activity

To determine the mechanisms underlying PGE$_2$-dependent regulation of *FOXP3*, we analyzed the expression of *FOXP3* mRNA and protein levels in untreated or PGE$_2$-treated (13 and 26 μM) Jurkat T cells in the presence or the absence of TCR-activating stimuli. We found that PGE$_2$ 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, PGE$_2$ induced the greatest increase in *FOXP3* in TCR-stimulated Jurkat T cells. Western blot analysis showed a similar pattern of expression (Fig. 4*B*). Non-activated Jurkat T cells, in fact, expressed FOXP3 protein that was significantly up-regulated by both PGE$_2$ concentrations tested (13 and 26 μM). However, maximum FOXP3 up-regulation was obtained in Jurkat T cells stimulated with PGE$_2$ (26 μM) and activated with PMA plus ionomycin (Fig. 4*B*). 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 PGE$_2$ and costimulatory signals (Fig. 4*C*). This suggests that PGE$_2$ operates to induce *FOXP3* at the level of mRNA transcription.

Discussion

Several in vitro studies have reported an immunosuppressive role of PGE$_2$ in T cell proliferation. Most studies focused on the direct effect of PGE$_2$ 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 PGE$_2$-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 PGE$_2$ 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 naive CD4$^+$ T cells in response to a variety of stimuli (6–12).

In this study we report a novel mechanism of PGE$_2$-induced immunosuppression that occurs through the modulation/induction of human T reg cell function. Our results show that PGE$_2$ 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 PGE$_2$ exposure conferred acquisition of *FOXP3* expression consistent with the phenotypic feature seen in naturally occurring T reg cells. The original CD25^{dim} population became CD25^{bright} upon anti-CD3 activation. However, PGE$_2$ exposure markedly down-regulated the surface expression of CD25 in these CD3-stimulated CD4$^+$CD25$^-$ T cells that typically developed CD25^{high} 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 PGE$_2$. 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 III. *CD25 expression in CD4$^+$CD25^{bright} Treg cells treated with or without PGE$_2$[a]*

	24 h		48 h	
Sample No.	CD4CD25^{bright}	CD4CD25^{bright} PGE$_2$	CD4CD25^{bright}	CD4CD25^{bright} PGE$_2$
		Percentage positive and MFI		
I	87 (179)	70 (60)	79 (256)	69 (52)
II	89 (78)	79 (41)	84 (101)	47 (37)
III	94 (66)	84 (44)	97 (76)	28 (45)
IV	81 (45)	68 (39)	87 (56)	74 (28)

[a] CD25 surface expression and PGE$_2$-mediated modulation of CD25 in CD4$^+$ PBL. Data summarize ($n = 4$) the analysis of CD25 expression in CD4$^+$CD25^{bright} 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$^+$CD25^{bright}, pretreated with or without PGE$_2$ 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.
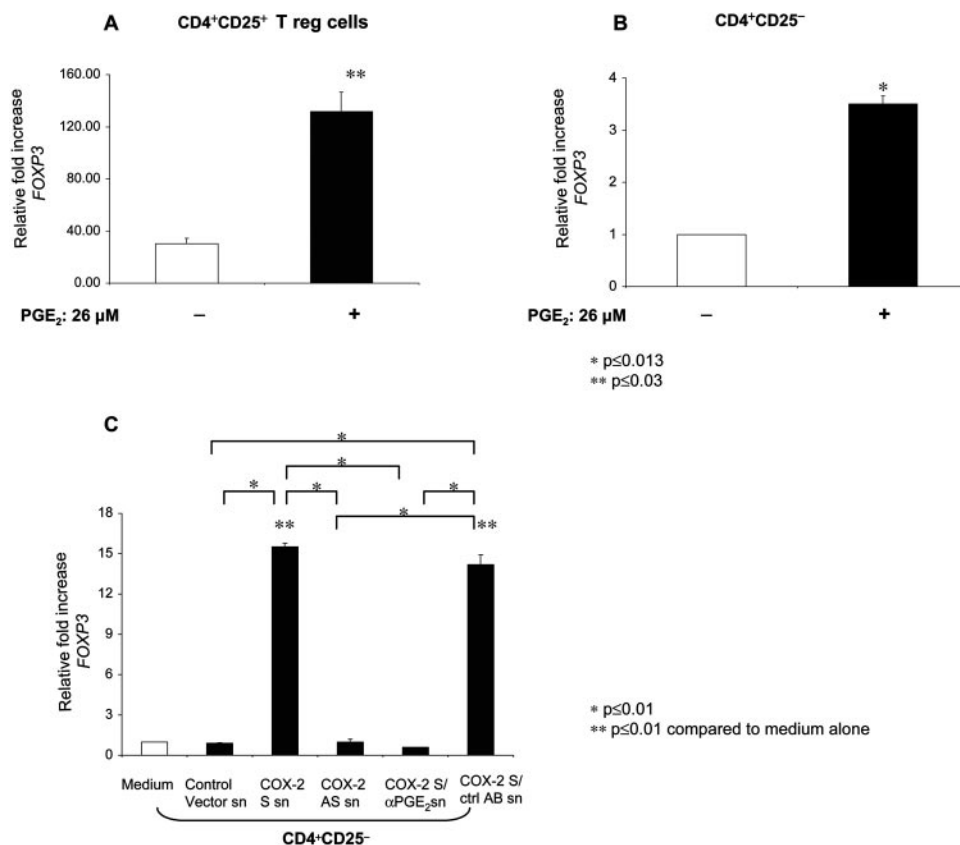
FIGURE 3. PGE$_2$ induces *FOXP3* mRNA expression in both CD4$^+$CD25$^+$ T reg cells and CD4$^+$CD25$^-$ T cells. Purified CD4$^+$CD25$^+$ T reg cells (*A*) and CD4$^+$CD25$^-$ T cells (*B*) were incubated without (−) or with (+) PGE$_2$ (26 μM) for 24 h. *C*, Alternatively, CD4$^+$CD25$^-$ T cells 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 *FOXP3* mRNA expression 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 was relative to *FOXP3* mRNA expression in CD4$^+$CD25$^-$. In PGE$_2$-treated CD4$^+$CD25$^-$ cells (*B*), the fold change in *FOXP3* mRNA was relative to that in untreated CD4$^+$CD25$^-$ cells. In CD4$^+$CD25$^-$ 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 ($\pm$SE) of one experiment of at least three performed in triplicate from different donors. Asterisks indicate statistical significance compared with the control value.

finding of PGE$_2$-mediated down-regulation of IL-2Rα is consistent with previous studies showing decreased IL-2 production and IL-2Rα expression in TCR-activated, PGE$_2$-stimulated T cells (37, 38, 42, 43). Thus, the PGE$_2$-mediated down-regulation of IL-2Rα may limit typical autocrine and paracrine IL-2-dependent function, such as the TCR-mediated proliferative response. Despite the reduced IL-2Rα expression, both PGE$_2$-treated CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$T cell subsets retained immunosuppressive capacities and exerted their suppressive functions in a cell contact independent manner.

The biochemical signals generated by the IL-2R are similar to those of the IL-7R (44). The receptors for these cytokines in fact use shared components such as the common γ-chain that appears to be essential for their mitogenic signals (44, 45). It has been shown that IL-2 signaling via STAT5 is required for T reg cell development, and yet this transcription factor is activated by IL-7 (45). In contrast, it has also been shown that, as members of the γ-chain family, IL-2R and IL-7R activate distinct signal-transducing factors, which may be differentially recruited to the receptor complex by their ligand-specific unit (45, 46). In the current study, addition of exogenous IL-7 overcame the suppressive capacity of naturally occurring CD4$^+$CD25$^+$ T reg cells and partially neutralized the inhibition of proliferation mediated by the PGE$_2$-induced regulatory T cells (CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$). Exogenous IL-2, however, had no ability to counteract the observed sup-

pressed proliferation. The PGE$_2$-induced IL-2Rα down-regulation may affect T cell IL-2 responsiveness, and perhaps higher concentrations of IL-2 may be required to 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.

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, PGE$_2$-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 in vitro regulatory function, because Ab-blocking studies could not reverse the inhibitory effect of either PGE$_2$-treated CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells (data not shown). Taken together, our data indicate that PGE$_2$-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,
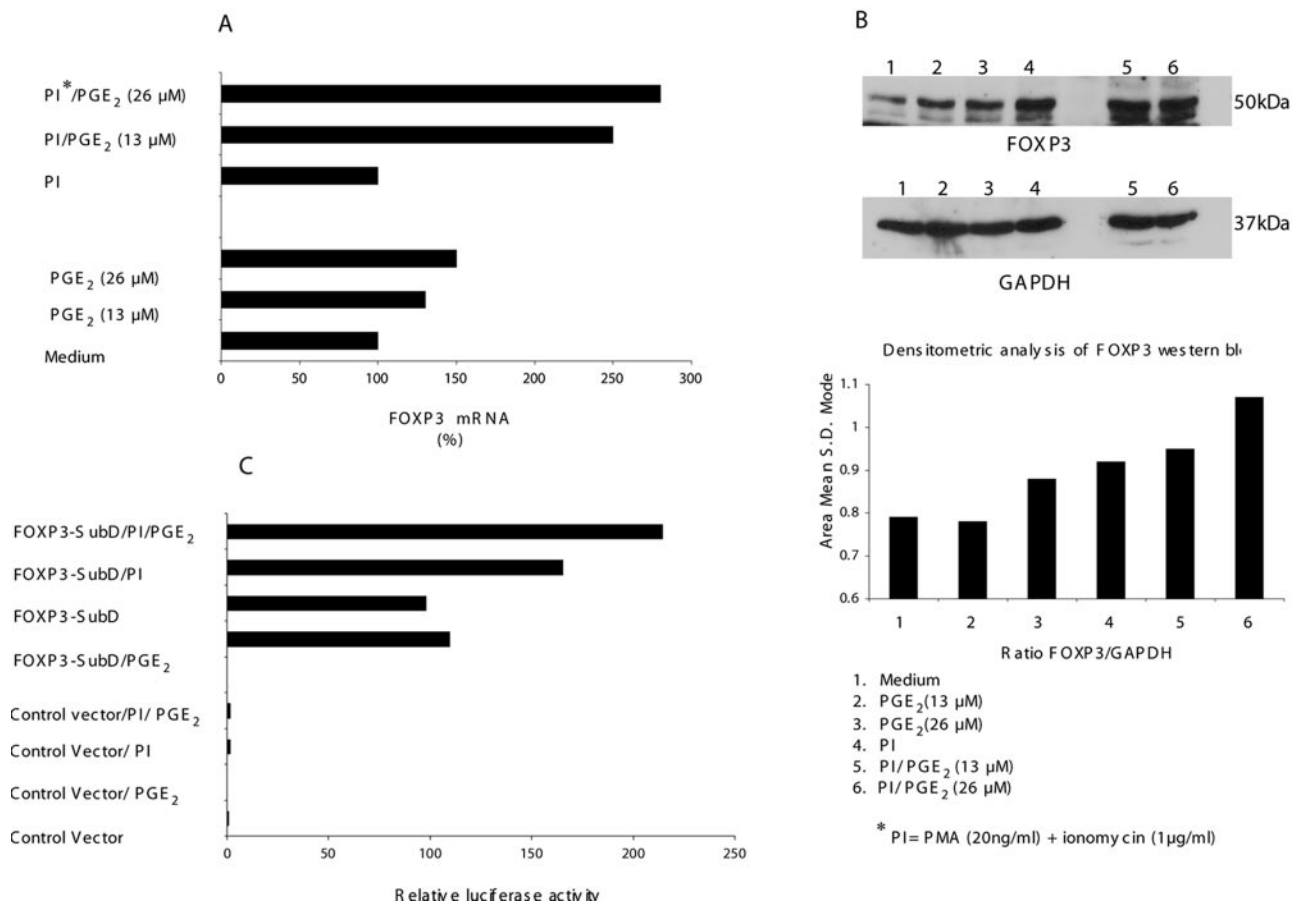
FIGURE 4. PGE$_2$ enhances *FOXP3* mRNA expression, gene transcription, and protein. *A*, Jurkat T cells were activated for 18 h as indicated. *FOXP3* mRNA expression was quantified by real-time PCR as described in *Materials and Methods. B, Top panel*, FOXP3 protein was analyzed by Western blot after 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*, PGE$_2$ induces *FOXP3* promoter activity. Jurkat T cells cotransfected with *FOXP3* promoter and firefly luciferase reporter gene constructs (*FOXP3*-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.

glucocorticoid-induced TNFR family-related protein, and CTLA-4, which may be more generally expressed by activated T cells (35, 36). In our study, PGE$_2$-dependent T reg differentiation was indeed associated with induction of the transcription factor *FOXP3*, whose expression was also significantly up-regulated by PGE$_2$ in purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. Consistent with previous reports in which TGF-β was investigated (6–8), PGE$_2$-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 PGE$_2$ in modulating T reg cell function and differentiation. In conditions of PGE$_2$ 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 PGE$_2$ 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 PGE$_2$-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 PGE$_2$ production may, in turn, modulate T reg cell development and function. These findings suggest new therapeutic strategies targeting COX-2/PGE$_2$ in the prevention and treatment of cancer. This could include addition of COX-2 inhibitor treatment to immune-based therapies.

Acknowledgments

Disclosures

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