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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.

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[3] 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 $CD4^+C25^+$ 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-acti*n 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. 1*A*). 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. 1*A*). 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. 1*B*). 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

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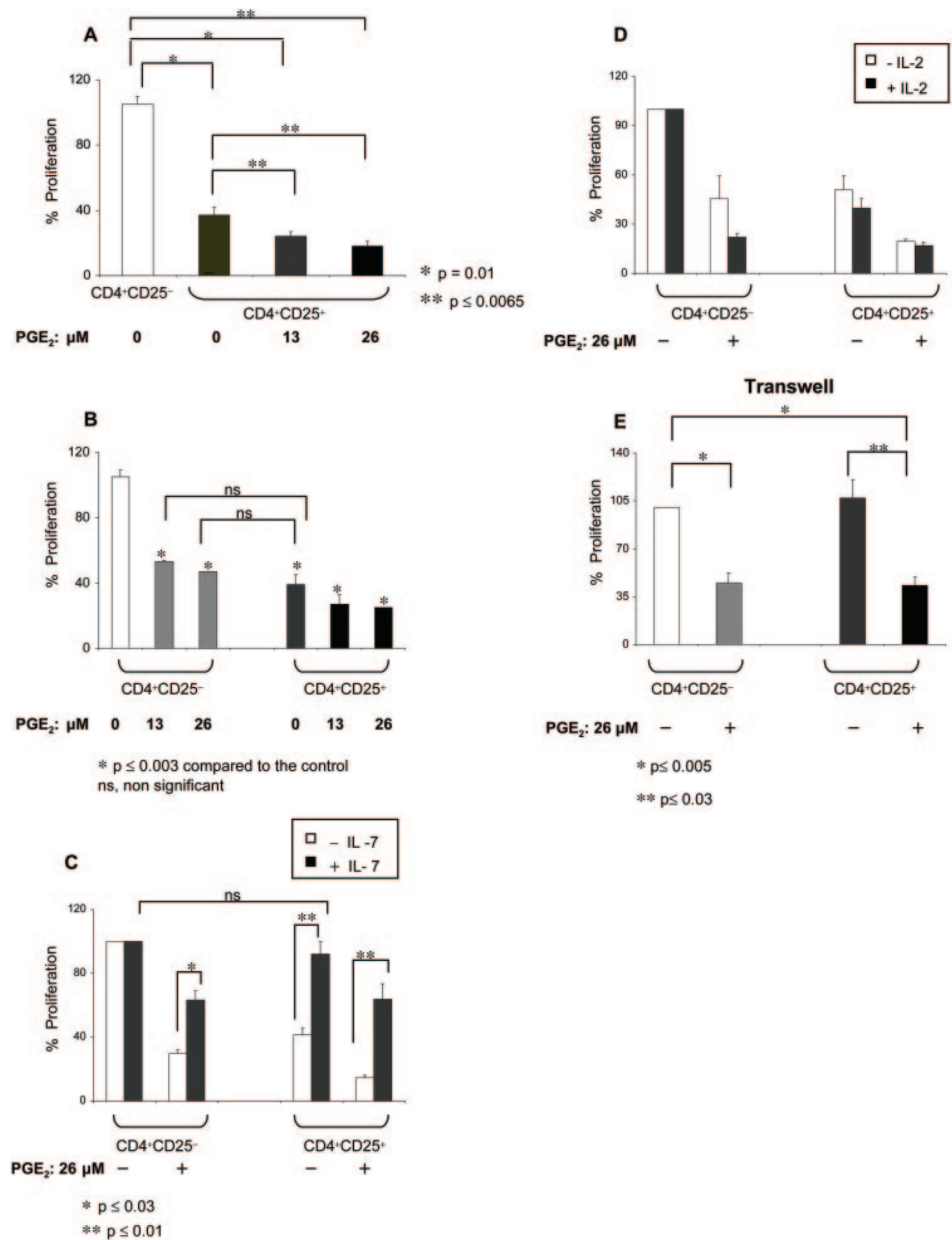

FIGURE 1. *A*, PGE_2 enhances T reg cell function. Purified $CD4^+CD25^+$ and $CD4^+CD25^-$ cells, precultured with or without PGE_2, were mixed with autologous $CD4^+$ cells in anti-CD3-bound, 96-well plates for 5 days. *B*, PGE_2 induces T reg cell activity in $CD4^+CD25^-$ T cells. $CD4^+CD25^+$ and $CD4^+CD25^-$ 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 (±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 (≥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. 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.

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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. 2*A* 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. 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 PGE_2, 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 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-β1, 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. 3*A*). Furthermore, a 24-h preincubation with PGE_2 (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 PGE_2 (26 μM), *FOXP3* mRNA expression was also significantly induced, consistent with the acquisition of a T reg cell suppressive function (Fig. 3*B*). 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. 3*C*). Control Ab did not impact the COX-2 S tumor supernatant-mediated induction of *FOXP3* mRNA expression (Fig. 3*C*).

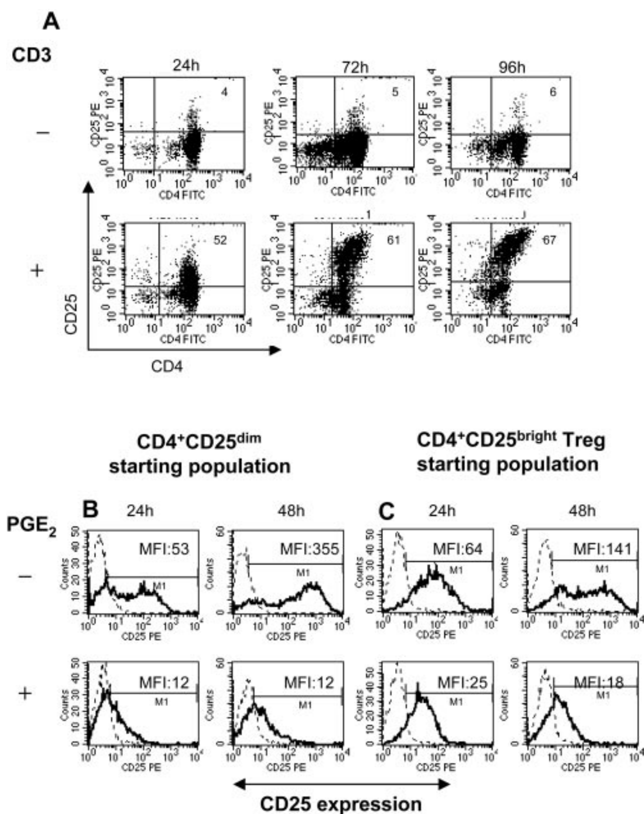

FIGURE 2. PGE_2 down-regulates CD25 expression in CD3-activated $CD4^+$ lymphocytes. *A*, PGE_2-treated, CD3-activated $CD4^+CD25^{dim}$ T cells up-regulate CD25 expression. Double-positive $CD4^+CD25^+$ cells were measured in unstimulated (*top panel*) and anti-CD3-stimulated (*bottom panel*), PGE_2-treated $CD4^+CD25^-$ 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 ($CD4^+CD25^+$). 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 (−; *top panel*) or with (+) PGE_2 (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 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.

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Table II. *CD25 expression in $CD4^+CD25^{dim}$ T cells treated with or without PGE_2*[a]

Sample No.	24 h		48 h		72 h		96 h	
	$CD4CD25^{dim}$	$CD4CD25^{dim}$ + 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]

Sample No.	24 h		48 h	
	$CD4CD25^{bright}$	$CD4CD25^{bright}$ 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.

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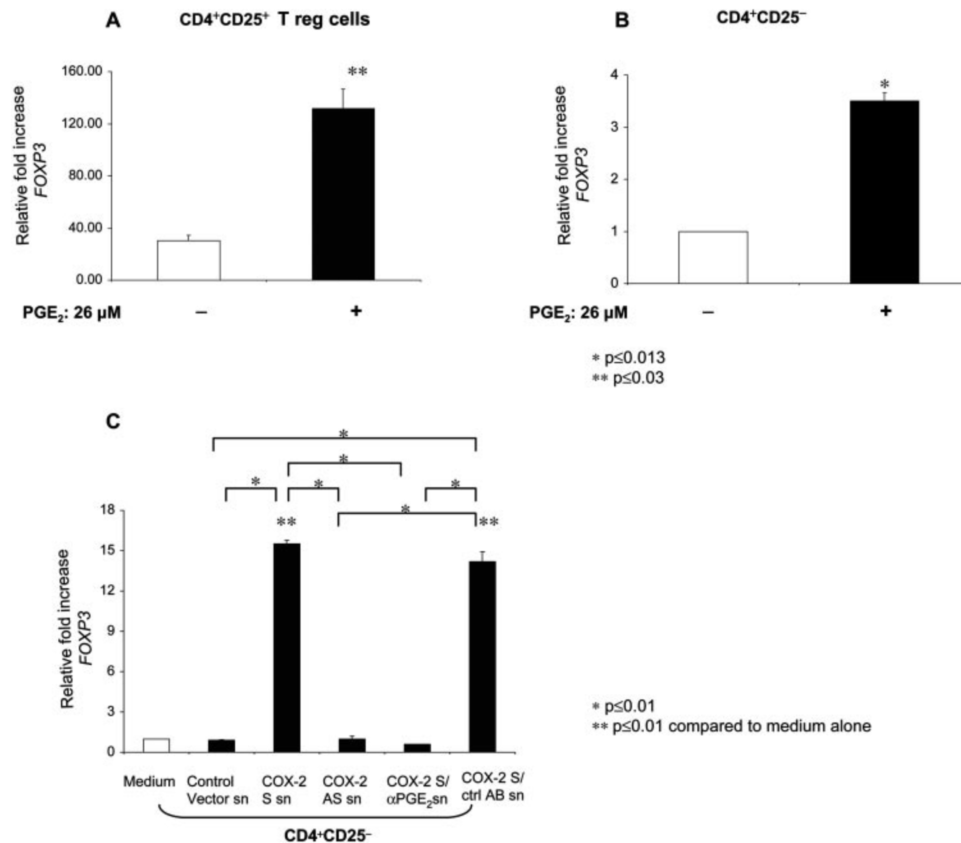

FIGURE 3. PGE_2 induces *FOXP3* mRNA expression in both $CD4^+CD25^+$ T reg cells and $CD4^+CD25^-$ T cells. Purified $CD4^+CD25^+$ T reg cells (*A*) and $CD4^+CD25^-$ T cells (*B*) were incubated without (−) or with (+) PGE_2 (26 μM) for 24 h. *C*, Alternatively, $CD4^+CD25^-$ T cells were cultured 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 (±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 suppressed 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,

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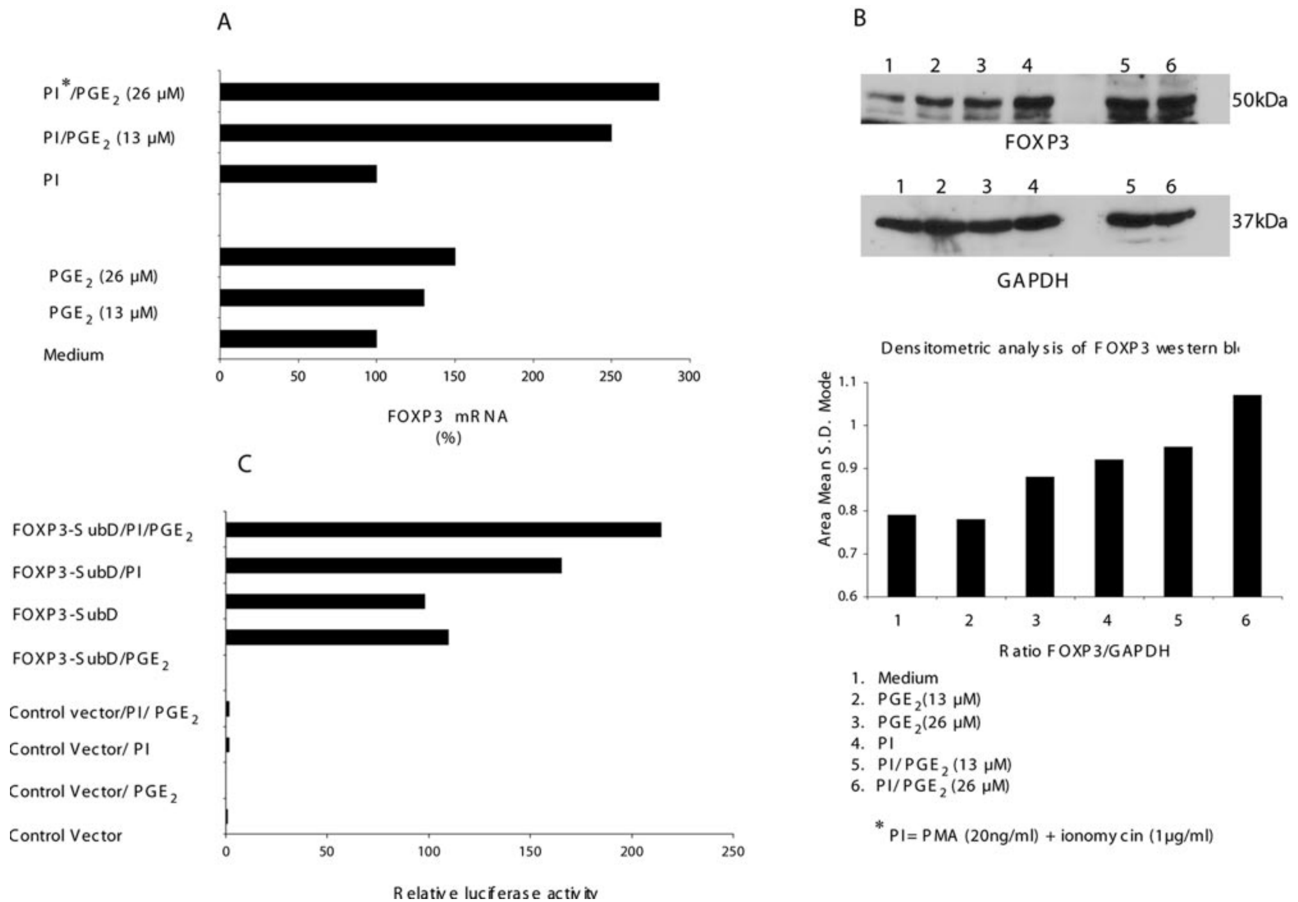

FIGURE 4. PGE_2 enhances *FOXP3* mRNA expression, gene transcription, and protein. *A*, Jurkat T cells were activated for 18 h as indicated. *FOXP3* mRNA expression was quantified by real-time PCR as described in *Materials and Methods. B*, *Top panel*, FOXP3 protein was analyzed by Western blot after 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.

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Disclosures

The authors have no financial conflict of interest.

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