

Expansion of Human T Regulatory Type 1 Cells in the Microenvironment of Cyclooxygenase 2 Overexpressing Head and Neck Squamous Cell Carcinoma

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Abstract

Cyclooxygenase 2 (COX-2) overexpression and production of prostaglandin E₂ (PGE₂) by head and neck squamous cell carcinomas (HNSCC) induce type 1 regulatory T (Tr1) cells and contribute to carcinogenesis by creating a tolerogenic milieu. To test this hypothesis, CD4⁺CD25⁻ T cells obtained from the peripheral blood of 10 normal donors were cocultured with autologous dendritic cells, irradiated HNSCC cells and cytokines, interleukin 2 (IL-2), IL-10, and IL-15. HNSCC cells were either COX-2 negative, constitutively expressed COX-2, were transfected with COX-2, or had COX-2 expression knocked down by small interfering RNA. Other modifications included coculture plus or minus the COX-inhibitor, Diclofenac, or synthetic PGE₂ in the absence of HNSCC. Lymphocytes proliferating in 10-day cocultures were phenotyped by flow cytometry, studied for cytokine production by ELISA and for suppressor function in CFSE inhibition assays plus or minus anti-IL-10 or anti-transforming growth factor- β_1 (TGF- β_1) monoclonal antibodies (mAb). COX-2⁺ HNSCC or exogenous PGE₂ induced outgrowth of Tr1 cells with the CD3⁺CD4⁺CD25⁻IL2R β ⁺IL2R γ ⁺FoxP3⁺CTLA-4⁺IL-10⁺TGF- β_1 IL-4⁻ phenotype and high suppressor functions (range, 46–68%). Small interfering RNA knockout of COX-2 gene in HNSCC led to outgrowth of lymphocytes with decreased IL2R γ ($P = 0.0001$), FoxP3 ($P = 0.05$), and IL-10 ($P = 0.035$) expression and low suppressor activity (range, 26–34%). Whereas COX-2⁺ cocultures contained IL-10 and TGF- β_1 (medians, 615 and 824 pg/mL), cytokine levels were decreased ($P < 0.0001$) in COX-2⁻ cocultures. Inhibition of COX-2 enzymatic activity in HNSCC abrogated outgrowth of Tr1 cells. Neutralizing mAbs to IL-10 and/or TGF- β_1 abolished Tr1-mediated suppression. COX-2 overexpression in HNSCC plays a major role in the induction of Tr1 cells in the tumor microenvironment. [Cancer Res 2007;67(18):8865–73]

Introduction

Chronic inflammation is increasingly often discussed as a critical component of tumor progression (1), but molecular mechanisms linking the two events remain largely unknown. Most epithelial malignancies are accompanied by overexpression of cyclooxyge-

nase 2 (COX-2), an inducible enzyme responsive to cytokines, growth factors, oncogenes, or tumor promoters during inflammation or cancer (2, 3). Its rapid induction results in enhanced synthesis of prostanoids, i.e., prostaglandin E₂ (PGE₂), in inflamed and neoplastic tissues (4). Elevated levels of PGE₂ at the tumor site have several procarcinogenic effects, including direct stimulation of tumor growth (5) and inhibition of immune surveillance (6). PGE₂ can also indirectly suppress dendritic cell functions and antitumor T-cell responses (7, 8). Recent studies have emphasized indirect effects of tumor-derived PGE₂ on induction and accumulation of different types of immune suppressor cells (9). PGE₂ has been shown to facilitate both expansion of FoxP3⁺CD4⁺CD25⁺ naturally occurring regulatory T (nTreg) cells (10) and induction of interleukin 10⁺(IL-10⁺)CD4⁺ type 1 regulatory T (Tr1) cells in the COX-2-positive microenvironment (11). Evidence that inhibition of COX-2 enzymatic activity can restore antitumor effects through antiangiogenic effects or by promoting tumor cell apoptosis exists (12, 13).

In this study, we have focused on the putative effects of tumor-derived PGE₂ on induction or modulation of the regulatory T cell subset Tr1. Tr1 cells were initially identified by their ability to produce certain cytokines, like IL-10 and transforming growth factor- β (TGF- β), but not IL-4 (14). Later reports suggested that Tr1 cells are induced from naive T-cell precursors and down-regulate immune responses via immunosuppressive cytokines IL-10 and TGF- β (15).

In the tumor microenvironment, a cross-talk between tumor cells, stromal cells, and lymphocytes may contribute to a proinflammatory cytokine repertoire, and tumor-derived factors, such as PGE₂, nitric oxide (16), and others, may influence signaling pathways in immune cells (17). Signals generated in the tumor microenvironment may result in activation of T lymphocytes and may promote their differentiation into a lymphocyte subset with regulatory functions. It is possible that this mechanism is exploited by tumors to induce immune tolerance and promote carcinogenesis (18).

Recently, we established a Tr1 *in vitro* assay (IVA) system, in which it was possible to induce Tr1 cells from naive T-cell precursors in the peripheral blood of normal donors by coculturing them with autologous immature dendritic cells (iDC) and tumor cells in the presence of low doses of IL-2 (10 IU/mL), IL-10 (20 IU/mL), and IL-15 (20 IU/mL; ref. 19). We confirmed the Tr1 phenotype and suppressor functions of the generated T cells. To further evaluate mechanisms used by the tumor to escape from immunosurveillance, we now modified this *in vitro* system by including head and neck squamous cell carcinoma (HNSCC) cells, which expressed or were genetically engineered to express COX-2. Cocultures in which HNSCC

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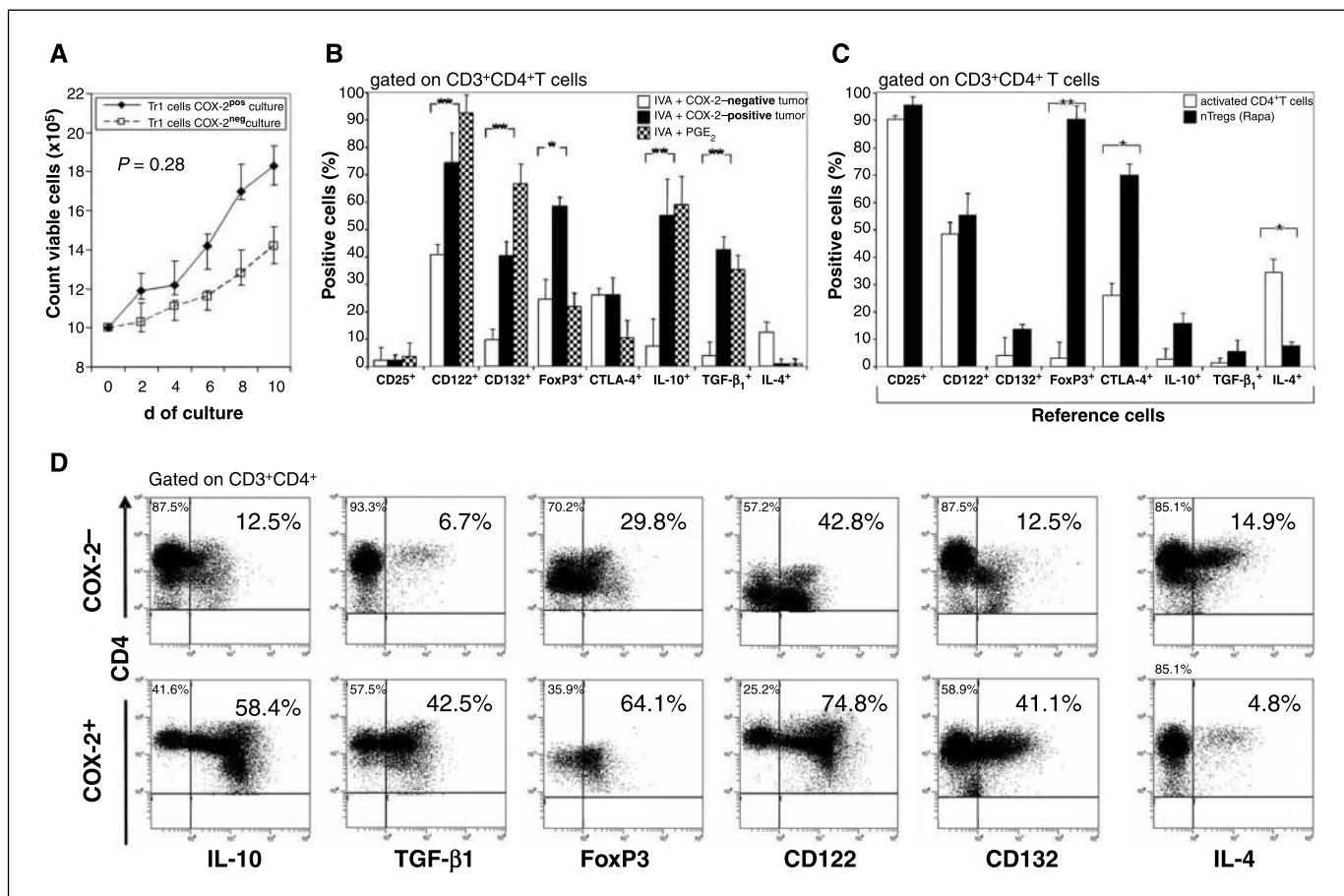


Figure 1. Phenotypic characteristics of $CD4^+CD25^-$ T cells cocultured with tumor cells or synthetic PGE_2 in the IVA model. **A**, numbers of proliferating $CD4^+CD25^-$ T cells cocultured with autologous iDC, cytokines, and tumor cells either positive (solid line) or negative (interrupted line) for COX-2 at different time points. Points, mean values from six experiments done with cells of three normal donors; bars, SD. **B**, effects of COX-2-positive PCI-1 and PCI-13 (■) or COX-2-negative ANT-1 (□) HNSCC cells on $CD4^+CD25^-$ T cells cocultured for 10 d with Tr1 cytokines and autologous iDC. The effects of synthetic PGE_2 (26 μ mol/L) replacing tumor cells are also shown (▣). These coculture conditions simulate tumor microenvironment. **C**, phenotypic characteristics of $CD3^+CD4^+$ T cells in reference cocultures. Reference cells are nTreg generated from MACS-separated $CD4^+CD25^-$ T cells and cultured for 10 d as described in Materials and Methods (■) and activated $CD4^+CD25^-$ T cells cultured in presence of IL-2 alone for 10 d (□). Columns, mean percentage (B and C) of positive cells from experiments done with cells of 10 different normal donors; bars, SD. Asterisks indicate significant differences (*, $P < 0.05$ and **, $P < 0.01$). **D**, flow cytometry dot plots showing relative expression of selected markers on T lymphocytes cultured in the Tr1 IVA for 10 d in the presence of tumor cells negative (top) or positive (bottom) for COX-2 expression. Gates were set on $CD3^+CD4^+$ cells. The data are representative for cells generated in IVA out of 10 done with cells of different donors.

did not express COX-2 or had inhibited COX-2 enzymatic activity were used as controls. Phenotypic and functional profiles of lymphocytes exposed to COX-2⁺ tumor cells indicated that Tr1-cell generation in the tumor microenvironment is linked to COX-2-mediated mechanisms.

Materials and Methods

Tumor cell lines. PCI-1 and PCI-13 are HNSCC cell lines established from primary tumors and maintained in our laboratory at the University of Pittsburgh as previously described (20). The spontaneous cell line ANT-1 was established from a HNSCC patient and maintained in our laboratory at the University of Munich in Germany. The tumor cell lines were maintained in DMEM supplemented with 10% Δ FCS, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine at 37°C in an atmosphere of 5% CO_2 in air. All cell lines were routinely tested for *Mycoplasma* and were found to be negative.

COX-2 small interfering RNA transfection. The HNSCC cell lines PCI-1 and PCI-13 constitutively overexpress COX-2 as confirmed by immunoblotting. The expression of COX-2 in HNSCC cells was temporarily silenced

using small interfering RNA (siRNA). Transfections with siRNA were conducted according to the manufacturers' recommendations (Santa Cruz Biotechnology, Inc.). Briefly, 2×10^5 PCI-13 cells were seeded in wells of six-well plates and adhered for 24 h in antibiotics-free RPMI medium. Cells were resuspended in transfection medium containing transfection reagent and COX-2 siRNA (human) at different concentrations or negative control siRNA. Cells were incubated for 6 h at 37°C in an atmosphere of CO_2 in air, then supplemented with the same volume of complete RPMI medium and incubated for additional 24 h. This medium was replaced by the complete DMEM growth medium for titration experiments or for IVA cultures to generate Tr1. Control siRNA used in these experiments had no homology to known sequences from humans (nontargeting 20–25 nucleotide siRNA). The optimal concentration of COX-2 siRNA (200 nmol/L) was determined by titration experiments until COX-2 expression was successfully silenced during 10 days of culture. Expression of the targeted gene was tested on days 3, 5, 8, and 10, using Western blots for COX-2 protein expression and enzyme immunoassays (EIA) for PGE_2 production.

Stable COX-2 transfection. ANT-1 cells are constitutively negative for COX-2 expression and were transfected with expression plasmids encoding cytomegalovirus-driven COX-2 (a kind gift from Timothy Hla, University of Connecticut). The empty pcDNA3.1 vector was transfected as control.

Clones were selected with hygromycin, and *COX-2* expression was tested by reverse transcription-PCR (RT-PCR) and Western blot analysis, whereas PGE_2 production was tested by EIA.

IVA. Peripheral blood mononuclear cells (PBMC) obtained from 10 normal donors were processed for generation of Tr1 cells as previously

described (19). Briefly, PBMC were isolated on Ficoll-Hypaque density gradients (GE Healthcare Bio-Sciences Corp.) and separated into monocytes and lymphocytes via plastic adherence. Monocytes were differentiated into iDCs by culturing in AIM V supplemented with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF; Berlex) for 7 days.

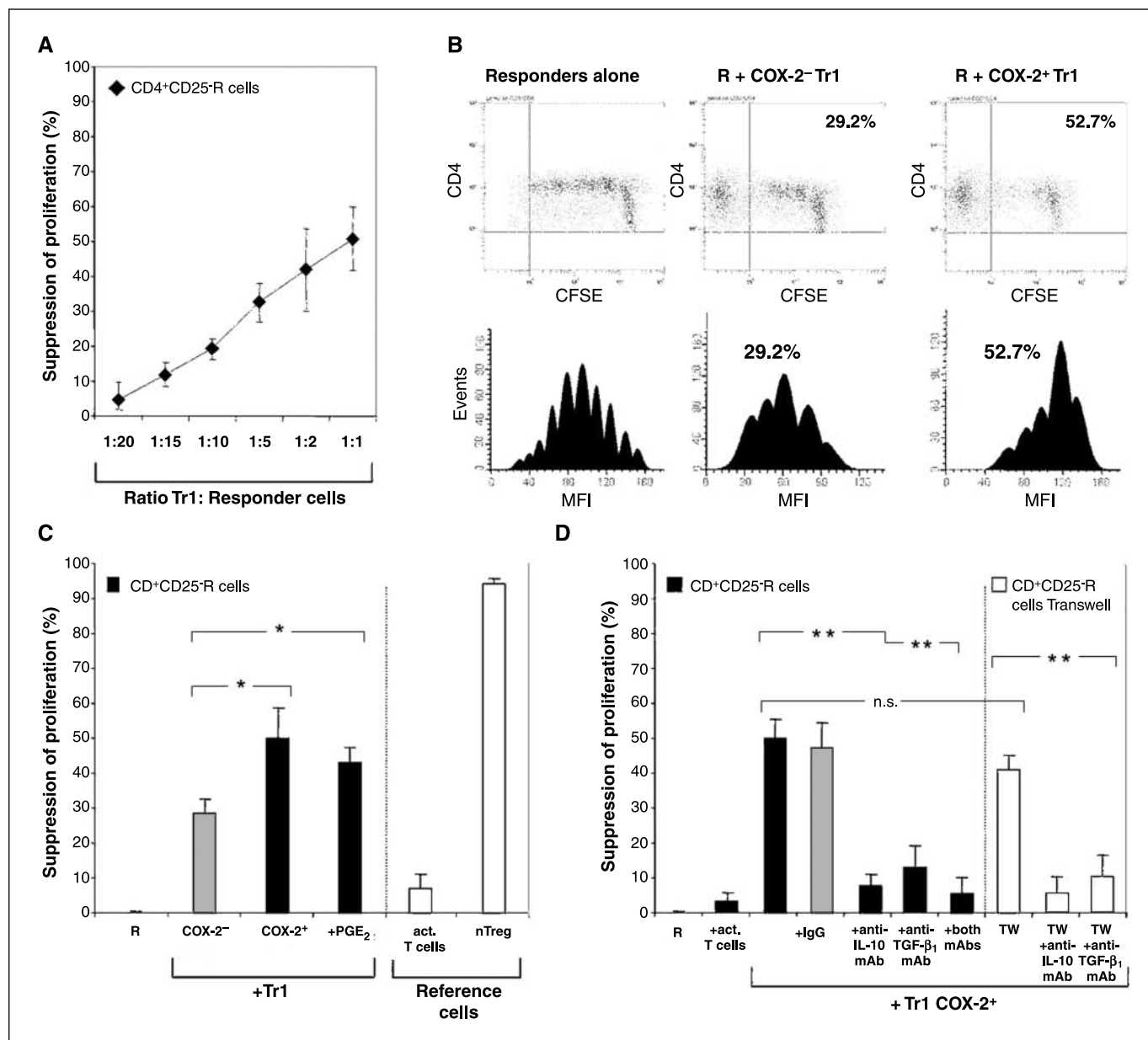


Figure 2. Suppressor activity of $CD4^+CD25^-$ T cells cocultured with tumor cells, PGE_2 , or rapamycin. **A**, Titration of Tr1 cells (S) into CFSE-labeled proliferating R cells. *Points*, mean percentage suppression of proliferation at different S/R ratios derived from experiments with cells of three different donors; *bars*, SD. **B**, proliferation data. Dot blots (*top*) show proliferation of $CD4^+CFSE^+$ R cells upon $CD3/CD28$ stimulation \pm autologous $CFSE^-$ S cells after 5 d of coculture. S cells were obtained from $COX-2^-$ or $COX-2^+$ Tr1 IVA and added to R cells at the start of the culture at the 1:1 ratio. The acquisition gate was restricted to lymphocytes, as determined by characteristic forward- and side-scatter properties of the cells. Analysis gates were restricted to the $CD3^+CD4^+$ T cell and $CD4^+CFSE^+$ T cell subsets. Percentages indicate proliferation of $CFSE^+$ R cells. Corresponding histograms (*bottom*) show ModFit-generated profiles of suppression of proliferation of CFSE-labeled R cells. The program calculates the percentage of suppression induced by S cells. The results are from one representative experiment out of 10 done with cells of different donors. **C**, MACS-purified CFSE-labeled $CD4^+CD25^-$ R cells were $CD3/CD28$ mAb stimulated and cultured as described in Materials and Methods. Lymphocytes (S) from the autologous Tr1 IVA containing $COX-2^-$ (■) tumor cells, $COX-2^+$ (■) tumor cells, or synthetic PGE_2 (■) were added at the start of the culture at the 1:1 ratio. Reference cells (□; activated T cells or nTreg) were similarly cocultured with autologous CFSE-labeled R cells. **D**, suppression by Tr1 cells of proliferating R cells was reduced when neutralizing anti-IL-10 and/or anti-TGF- β_1 mAb (but not isotype control IgG) were added to cocultures. R cells (CFSE-labeled autologous $CD4^+CD25^-$ T cells) were stimulated with $CD3/CD28$ mAb and cultured for 5 d. S cells (Tr1) were generated in IVA with $COX-2^+$ HNSCC. Tr1 cells (used as controls), but not activated T cells, mediated suppression when they were cocultured with R cells in the presence or absence of transwell (TW) inserts. Addition of anti-IL-10 and anti-TGF- β_1 mAb in the presence of transwell inserts significantly reduced levels of suppression. *Columns*, mean percentage of suppression of proliferation (**C** and **D**) of CFSE-labeled autologous $CD4^+CD25^-$ T cells observed in experiments done with cells of 10 different normal donors; *bars*, SD. Asterisks indicate significant differences (*, $P < 0.05$ and **, $P < 0.01$).

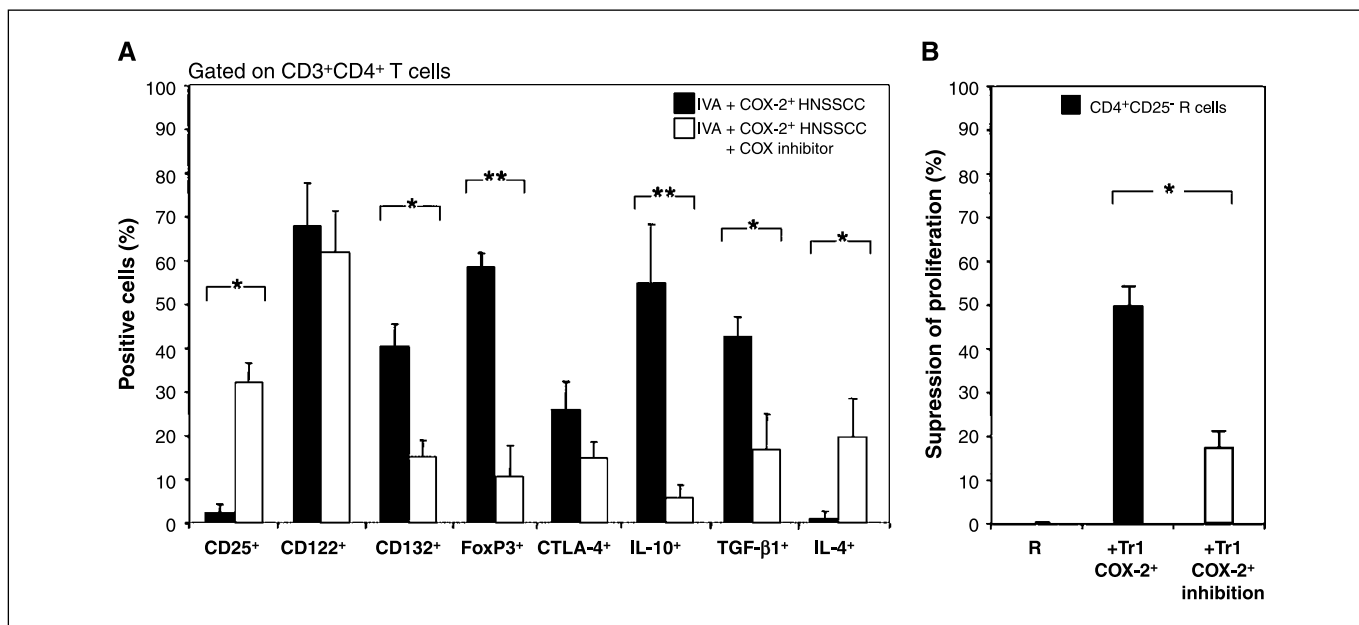


Figure 3. Phenotypic and functional analyses of Tr1 cells cultured in the presence of COX-2⁺ tumor cells and Diclofenac, a generic COX inhibitor. **A**, effects of Diclofenac (1.5 μmol/L) on MACS-isolated CD4⁺CD25⁻ T cells cultured in COX-2⁺ Tr1 IVA for 10 d (□). As a control, CD4⁺CD25⁻ T cells were cultured in COX-2⁺ Tr1 IVA without Diclofenac (■). *Columns*, mean percentage of positive cells from experiments done with cells of 10 different normal donors; *bars*, SD. Flow cytometry analysis was done with gates set on CD3⁺CD4⁺ T cells. Asterisks indicate significant differences (*, $P < 0.05$ and **, $P < 0.01$). **B**, a comparison of suppressor activity of lymphocytes generated in the Tr1 IVA containing COX-2⁺ tumor cells and Diclofenac (□) or no inhibitor (■) on proliferation of CFSE-labeled R cells. Cultured cells were added at the start of the culture (ratio, 1:1) and coincubated with autologous CFSE-labeled R cells for 5 d as described in Materials and Methods. *Columns*, mean percentage of suppression of proliferation of CFSE-labeled CD4⁺CD25⁻ T cells observed in experiments with cells of 10 different normal donors; *bars*, SD.

CD4⁺CD25⁻ T cells were isolated using regulatory T cell separation kit and AutoMACS (Miltenyi Biotech). T cells were coincubated with autologous iDC and tumor cells in AIM V medium in duplicate wells of 24-well plates for 10 days. IL-2 (10 IU/mL), IL-10 (20 IU/mL), IL-15 (20 IU/mL; Peprotech) and, where specified, rapamycin (1 nmol/L; Sigma) were added on days 0, 3, and 6. On day 9, culture medium was replaced by fresh medium, containing anti-CD3 antibody (1 μg/mL; American Type Culture Collection) and Brefeldin A (1 μg/mL; Sigma). On day 10, lymphocytes and culture medium were harvested.

Tr1 IVA cultures were varied by using tumor cells, which were constitutively either positive (PCI-1, PCI-13) or negative for COX-2 (ANT-1). In some experiments, synthetic PGE₂, instead of tumor cells, was used. PGE₂ was pretitrated to determine its optimal working concentration (26 μmol/L). Furthermore, in some experiments, tumor cells were used, where either COX-2 expression in COX-2-positive tumor cell lines was temporarily knocked down using siRNA or where cell lines, which were constitutively negative for COX-2, were transfected to express COX-2.

Also, a nonspecific COX inhibitor, Diclofenac sodium salt (Sigma), was added to some Tr1 IVA, which contained COX-2⁺ HNSCC, on days 0, 3, 6, and 9. Diclofenac was initially pretitrated into COX-2⁺ Tr1 IVA to determine its optimal working concentration (1.5 μmol/L).

Human nTreg and activated CD4⁺ T cells ("reference cells") were used for comparisons with Tr1 cells. nTreg cells were cultured as previously described (21). Briefly, MACS-isolated CD4⁺CD25⁺ T cells (10⁶) from PBMC of normal donors were cultured with anti-CD3/anti-CD28 antibody-coated magnetic beads (T-cell expansion kit) in the presence of 1,000 IU/mL IL-2 and rapamycin (1 nmol/L). Activated CD4⁺ T cells were expanded starting with MACS-isolated CD4⁺CD25⁻ T cells (10⁶) from PBMC of normal donors by culture in the presence of 1,000 IU/mL IL-2. Reference cells were cultured for 10 days in a complete medium, in wells of 24-well plates at 37°C in an atmosphere of 5% CO₂ in air. Cells and supernatants were harvested on day 10 and used for phenotypic, functional, and cytokine analyses.

Flow cytometry. Cells from IVA or reference cultures were stained for flow cytometry as previously described (19). Briefly, cells were stained for

surface markers and fixed with 2% (v/v) paraformaldehyde in PBS. Next, cells were permeabilized by incubating them in saponin (0.1% v/v in PBS) for 30 min at 37°C in an atmosphere of 5% CO₂ in air. Antibodies (Ab) for intracellular staining were added, and cells were incubated for additional 25 min at 4°C in the dark. The following Abs were used for intracellular staining: anti-FoxP3-FITC, anti-CD152-PE, anti-IL-10-PE, anti-TGF-β1-FITC and anti-IL-4-FITC. CD4⁺CD25⁻ T cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1 ng/mL) and ionomycin (1 μmol/L) for 6 h in the presence of Brefeldin A (1 μg/mL) to serve as a positive control for intracellular staining. Appropriate isotype controls were used in all experiments. The following monoclonal antibodies (mAb) were purchased from Beckman Coulter: purified anti-CD28 mAb, anti-CD3-ECD, anti-CD4-PC5, anti-CD25-FITC, anti-CD25-PE, anti-CD122-FITC, anti-CD132-PE, anti-CD152-PE, and isotype controls IgG1, IgG2a, and IgG2b. Anti-IL-10-PE and its isotype control PE rat IgG2a_k were purchased from BD Pharmingen; anti-FoxP3-FITC and anti-IL-4-FITC were from eBioscience; anti-TGF-β1-FITC (clone TB21) were from Antigenix America, Inc. Flow cytometry was done using a FACScan flow cytometer (Beckman Coulter) equipped with Expo32 software. Lymphocytes were identified by forward- and side-scatter gating profiles, and 10⁵ cells were acquired for analysis. Furthermore, analysis gates were restricted to the CD3⁺CD4⁺ T-cell subset. Data were analyzed using Coulter EXPO 32v1.2 analysis software.

CFSE-based suppression assay. Responder CD4⁺CD25⁻ T cells (R) were MACS-isolated from PBMC, stained with 1.5 μmol/L CFSE (Molecular Probes/Invitrogen), and cocultured with autologous suppressor cells (S) as previously described (19). Briefly, R cells were stimulated with plate-bound anti-CD3 mAb (1 μg/mL) and soluble anti-CD28 mAb (1 μg/mL) in complete AIM V medium containing IL-2 (150 IU/mL) in wells of 96-well plates (5 × 10⁵ per well). T cells (S) obtained from 10 days IVA or reference cultures were added to R cells at different ratios (i.e., 1S:1R, 1S:5R, 1S:10R) and cocultures were incubated for 5 days at 37°C in an atmosphere of 5% CO₂ in air. Flow cytometry analysis and CFSE data analysis were done using ModFit LT for Win32 software provided by Verity Software House, Inc. T-cell populations were classified as suppressive if they inhibited proliferation of

autologous CD4⁺CD25⁻ R cells in the coculture assay and if decreasing the number of Tr1 cells relative to the number of CD4⁺CD25⁻ R cells in coculture restored proliferation. Tr1 cell populations that satisfied both of these criteria were classified as suppressor T cells. These criteria were applied to all populations tested.

To test the role of IL-10 and TGF-β₁ in Tr1-mediated suppression, neutralizing Abs to IL-10 and/or TGF-β₁ (R&D) or isotype control IgG were added to cocultures. The mAbs were pretitered and were added to the assays at the final concentration of 2 μg/mL or 20 ng/mL, respectively. Cells were cultured and analyzed as described above.

In additional CFSE assays, R cells were plated at 10⁶ cells per well in wells of 24-well plates (Corning) coated with anti-CD3 mAb (1 μg/mL) and

equipped with 6.5-mm transwell inserts (pore size, 0.4 μm). Cells were cultured in complete AIM V medium containing IL-2 (150 IU/mL) and anti-CD28 mAb (1 μg/mL) for 5 days at 37°C in an atmosphere of 5% CO₂ in air. T cells obtained from 10 day IVA or from reference cultures were either plated in the bottom wells containing R cells or placed in the transwell insert at the 1:1 ratio. After the harvest, suppression of proliferation of CFSE-labeled R cells was analyzed using ModFit software as described above.

Western blot analysis. Whole-cell protein extracts were prepared either from 5 × 10⁵ HNSCC cells or from iDC which were cultured alone or supplemented with IL-2/IL-10/IL-15 and/or PGE₂ at the concentrations used in the Tr1 IVA for 24 h. The lysis buffer contained Halt protease

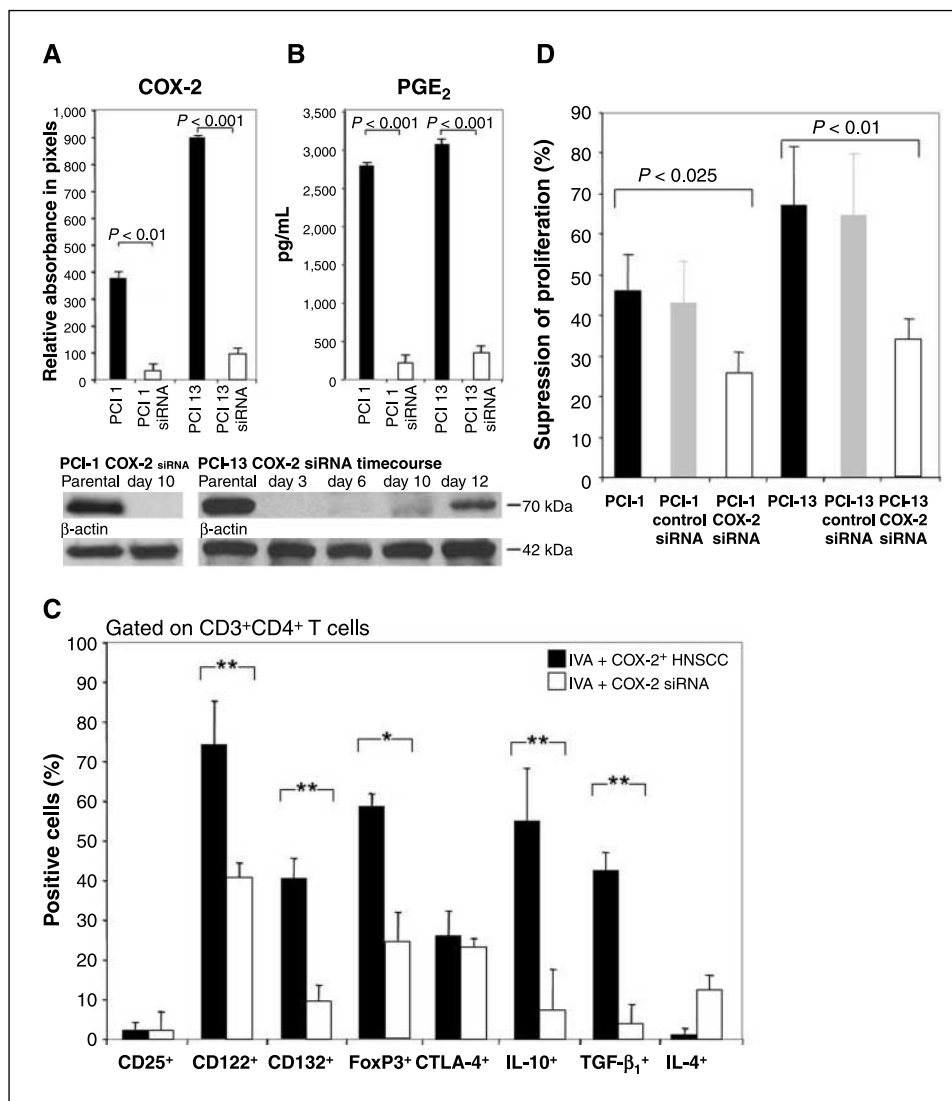


Figure 4. siRNA inhibition of COX-2 reduced outgrowth of functional Tr1 cells. **A**, immunoblot analysis for COX-2 protein expression of cell lysates prepared from 2 × 10⁶ HNSCC cells. Both lines were highly positive for COX-2 expression (*left blots*). COX-2 expression was temporarily silenced using COX-2-specific siRNA. Blots show protein expression of cells silenced for COX-2 on day 10 after RNA expression knockdown. Representative results from experiments done in triplicate are shown, comparing relative COX-2 expression in parental (■) and siRNA-modified tumor cells (□). Immunoblots show protein expression (*top*) and β-actin control for equal protein loading (*bottom*). *Left blots*, COX-2 expression in the parental PCI-1 cell line and its expression after siRNA inhibition on day 10; *right blots*, COX-2 expression in the parental PCI-13 cell line and time course of siRNA inhibition of COX-2 expression through day 12. **B**, levels of PGE₂ in supernatants of 5 × 10⁵ tumor cells: supernatants from HNSCC cell lines PCI-1 and PCI-13 (■) and siRNA-modified cells (□). *Columns*, mean (pg/mL) from triplicate experiments; *bars*, SD. **C**, effects of COX-2-specific siRNA knockdown in COX-2⁺ tumor cells on CD4⁺CD25⁻ T cells cocultured for 10 d with Tr1 cytokines and autologous iDC. Phenotype of Tr1 cells cultured in IVA containing COX-2⁺ tumor cells (■) versus T cells cultured in IVA containing siRNA-modified tumor cells (□). *Columns*, mean percentage of positive cells from experiments with cells of 10 different normal donors; *bars*, SD. Asterisks indicate significant differences (*, P < 0.05 and **, P < 0.01). **D**, functional analysis of T lymphocytes cultured in the Tr1 IVA in the presence of COX-2⁺ HNSCC cell lines PCI-1 and PCI-13 without (■) or with (□) inhibition of COX-2 expression through siRNA or treated with nontargeting siRNA control (▨). Suppression by Tr1 cells of R cell proliferation was tested in 5-d CFSE cultures as described in Materials and Methods. *Columns*, mean percentage of suppression of proliferation of CFSE-labeled autologous CD4⁺CD25⁻ T cells observed in experiments done with cells of 10 different normal donors; *bars*, SD.

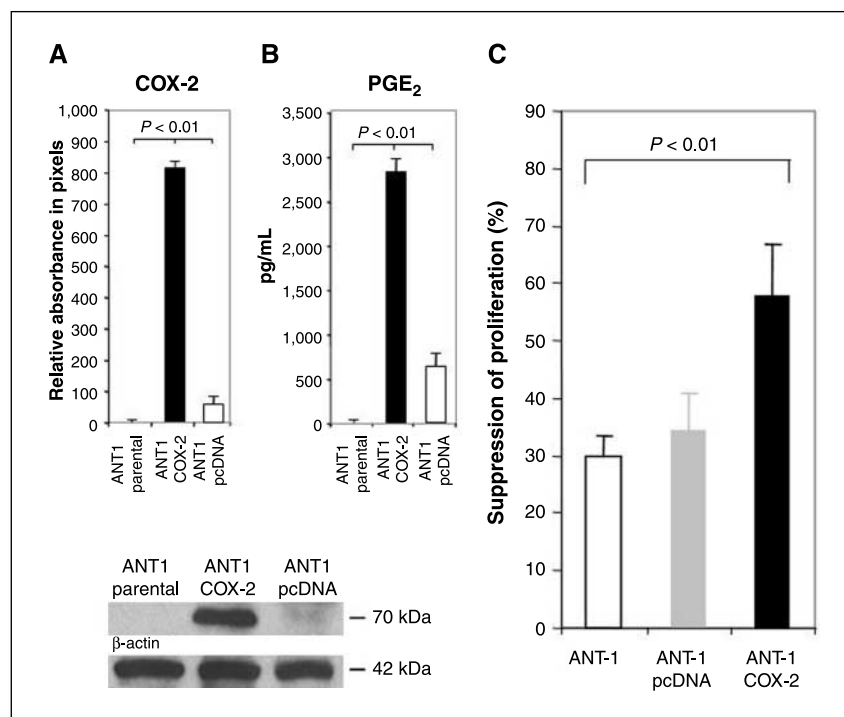


Figure 5. COX-2 transfection in HNSCC line and its effect on induction of Tr1 cells. **A**, immunoblot analysis for COX-2 protein expression in the cell lysate prepared from 2×10^6 HNSCC cells (ANT-1). ANT-1 cells, originally negative for COX-2 (parental; left blot) were transfected with COX-2 cDNA (middle blot) or with empty vector pcDNA (right blot). Immunoblots show COX-2 protein expression (top) and β -actin control for equal protein loading (bottom). **B**, levels of PGE₂ in supernatants of 5×10^5 tumor cells transfected (■) or not transfected (□) with COX-2 cDNA or with empty vector pcDNA. Columns, mean (pg/mL) from triplicate experiments; bars, SD. **C**, functional analysis of Tr1 cells cultured in Tr1 IVA in the presence of COX-2⁻ ANT-1 cells before (□) and after (■) COX-2 gene or pcDNA (■) transfection. Suppression by Tr1 cells of R cell proliferation was tested in 5-d CFSE cultures as described in Materials and Methods. Columns, mean percentage of suppression of proliferation of CFSE-labeled autologous CD4⁺CD25⁻ T cells observed in experiments done with cells of 10 different normal donors; bars, SD.

inhibitor (Pierce). Protein extracts were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. COX-2 was immunodetected using conjugated rabbit anti-human polyclonal antibody (concentration at 5 μ g/mL; Alpha Diagnostics, Inc.), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (concentration at 10 μ g/mL; Pierce). Protein bands on immunoblots were detected by enhanced chemiluminescence, followed by autoradiography. Equal protein loading was confirmed by using anti- β -actin Ab (clone AC-15; Sigma). Proteins were semiquantified by computerized densitometric analysis.

ELISA. Supernatants of 9-day IVA cocultures were removed and replaced by a fresh, complete medium containing anti-CD3 mAb (1 μ g/mL) but no exogenous cytokines. After 24 h, supernatants were collected and stored frozen. Supernatants of tumor cell lines were likewise replaced by fresh culture medium for 48 h, collected, and stored frozen. CD4⁺CD25⁻ T cells were stimulated with PMA (1 ng/mL) and Ionomycin (1 μ mol/L) for 6 h, and their supernatants served as controls. Levels of PGE₂, IL-4, IL-10, IL-12, and IFN- γ were determined by ELISA (purchased from R&D). Levels of TGF- β ₁ in acidified supernatants were also determined by ELISA. Nitric oxide was evaluated using the nitric oxide detection EIA (Assay Designs). Levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ , tumor necrosis factor- α (TNF- α), and GM-CSF were evaluated by Luminex technology, using a human cytokine 10-plex Ab bead kit (Biosource/Invitrogen). All assays have been done according to manufacturers' instructions.

Statistical analysis. All statistical analyses were done using the Student's *t* test, and *P* values of <0.05 were considered significant.

Results

Phenotypic characteristics of CD4⁺CD25⁻ T cells cocultured in different conditions. Overexpression of COX-2 and production of PGE₂ have been linked to immune suppression. We investigated the effects of tumor-derived PGE₂ on the induction and expansion of Tr1 cells. The previously described Tr1 IVA (19) was modified to include tumor cells positive or negative for COX-2 or PGE₂, respectively, and the assay was done with three different HNSCC cell lines. Parental cell lines were either positive (PCI-1, PCI-13) or

negative (ANT-1) for COX-2. Lymphocytes outgrowing in these cocultures were phenotyped and evaluated functionally. Coculture of CD4⁺CD25⁻ T cells with autologous iDCs and tumor cells in the presence of "Tr1 cytokines" (IL-2, IL-10, and IL-15) has led to the outgrowth of T lymphocytes with the phenotype that was significantly different (*P* < 0.01) from that of T cells in reference cultures (Fig. 1C). The outgrowing CD3⁺CD4⁺ cells were CD25⁻. They were CD122⁺CD132⁺FoxP3⁺CTLA-4⁻IL-10⁺TGF- β ₁⁺ and negative for IL-4 (Fig. 1B). As we have recently reported (19), this phenotype is characteristic for Tr1 cells, which appear in a microenvironment of solid tumors, and the requirement for tumor antigens, iDC and Tr1 cytokines to induce it was previously established.

In contrast, cocultures set up in the presence of tumor cells, which were negative for COX-2 expression (ANT-1) and produced no PGE₂, proliferated less well (Fig. 1A) and contained significantly fewer T cells positive for Tr1 markers, such as CD132 (IL2R γ), FoxP3, and CTLA-4 (Fig. 1B). These cells had lower marker intensity (Fig. 1D) and were also negative for CD25 and IL-4. Importantly, the percentages of cells positive for IL-10 and TGF- β ₁ (Fig. 1B and D) and the cytokine expression levels (Fig. 1D) were significantly decreased (*P* < 0.01) in these cultures.

When the coculture conditions were modified by replacing tumor cells with 26 μ mol/L of synthetic PGE₂, the phenotype of outgrowing lymphocytes was similar to that described for COX-2⁺ cocultures, except that the percentage of cells positive for IL2R γ was significantly higher (*P* < 0.05) and that of FoxP3⁺ cells was significantly lower (*P* < 0.01; Fig. 1B). Lymphocytes outgrowing in reference cultures (Fig. 1C) were CD25⁺ and FoxP3⁺, consistent with the nTreg phenotype (21), or were CD25⁺FoxP3⁻ (activated CD4⁺ T cells). Our results showed that T lymphocytes outgrowing in the COX-2⁻ Tr1 IVA were phenotypically quite distinct from nTreg or activated T lymphocytes (Fig. 1C).

Rapamycin augments outgrowth of lymphocytes with the Tr1 phenotype. We recently reported that the immunosuppressive

drug rapamycin enhances selective survival and expansion of both nTreg (21) and Tr1 cells (19). Cocultures containing COX-2⁺ tumor cells and rapamycin were enriched in IL-10⁺ Tr1 cells ($P < 0.01$) relative to cocultures set up with COX-2 negative tumor cells (data not shown). Also, in COX-2 negative Tr1 cocultures plus rapamycin, fewer outgrowing CD4⁺ T cells were IL-10⁺ ($P < 0.01$), although they did not express CD25 and contained fewer FoxP3⁺ cells and, thus, were distinct from lymphocytes in reference cultures (Fig. 1C). As expected, rapamycin was most effective in promoting expansion of Tr1 cells in the presence of COX-2⁺ tumor cells.

Suppressor activity of T cells with the Tr1 phenotype. As regulatory T cells have the ability to suppress proliferation of activated R cells, we evaluated suppression mediated by Tr1 cells generated *in vitro*. First, Tr1 cells outgrowing in the presence of COX-2⁺ or COX-2⁻ tumor cells were titrated into CFSE-labeled autologous CD4⁺CD25⁻ R cells at various suppressor (S) to R ratios. As shown in Fig. 2A, proliferation of R cells activated with plate-bound anti-CD3 and anti-CD28 mAbs was inhibited by Tr1 cells, linearly increasing with higher S/R ratios. Figure 2B shows that Tr1 cells, cocultured with COX-2⁺ tumor cells, mediated greater suppression than Tr1 cells generated in cocultures containing COX-2⁻ tumor cells, and the difference was significant at $P < 0.01$ (Fig. 2C). The Tr1 cells generated in cocultures containing exogenous PGE₂, instead of tumor cells, also mediated high levels of suppression (Fig. 2C). Tr1 cells cocultured with COX-2⁺ tumor cells or PGE₂ in the presence of rapamycin increased suppressive activity 1.5-fold (data not shown). Lymphocytes from reference cultures containing activated CD4⁺CD25⁻ T cells mediated no suppression, in contrast to CD4⁺CD25⁺ nTreg, which mediated excellent suppression (Fig. 2C).

Inhibition of IL-10 and TGF-β₁ and its effect on Tr1 suppressor activity. It has been suggested that Tr1 cells conduct their suppressive activity mainly through secretion of IL-10 and TGF-β₁ (22). To test this hypothesis, we added predetermined concentrations of neutralizing anti-IL-10 (2 μg/mL) and/or anti-TGF-β₁ (20 ng/mL) mAbs to suppression assays. As shown in Fig. 2D, neutralization of these cytokines resulted in a significant reduction of suppression mediated by Tr1 cells ($P < 0.01$) and concomitant increased proliferation of R cells. Assays done in the transwell system confirmed the key role of cytokines in Tr1 suppression. Tr1 cells, but not activated CD4⁺ T cells, were able to

suppress autologous R separated by the filter. Further, this suppression was blocked in the presence of neutralizing mAbs to IL-10 and TGF-β₁ (Fig. 2D).

Inhibition of COX-2 activity and its effect on Tr1 cell generation. To further evaluate the effect of COX-2 overexpression in tumor cells on Tr1 cell induction, the COX-2 pathway was inhibited using the generic COX inhibitor Diclofenac (1.5 μmol/L). T cells outgrowing in the presence of COX-2⁺ tumor cells plus the COX inhibitor were phenotypically distinct from control Tr1 cells generated in the absence of the inhibitor (Fig. 3A). They contained significantly fewer cells positive for CD132, FoxP3, and IL-10 ($P < 0.01$) but more cells positive for CD25 and IL-4. Functional analysis of T lymphocytes outgrowing in the presence of the COX inhibitor showed low levels of suppressor activity, compared with Tr1 cells generated in its absence (Fig. 3B).

siRNA-mediated inhibition of COX-2 expression in tumor cell lines. PCI-1 and PCI-13 are HNSCC cell lines positive for COX-2 expression (Fig. 4A) and produce PGE₂ (Fig. 4B). Both of these characteristics were temporarily silenced using siRNA specific for COX-2 (Fig. 4A and B). To confirm the successful silencing of the cell lines, protein expression of COX-2 in the parental and modified cell lines was determined, as was cytokine production. Supernatants of the silenced tumor cell lines were negative for IL-4, IL-12, TGF-β₁, or IL-10, and cytokines used in the Tr1 IVA had no effect on these cells (data not shown). In the IVA with the siRNA-modified tumor cells, outgrowing T lymphocytes showed a significantly reduced expression of IL-10, TGF-β₁, and CD132 relative to control IVA cultures (Fig. 4C). Suppressor activity of IL-10⁺CD4⁺ Tr1 cells outgrowing in the presence of the siRNA-modified tumor cells was also significantly reduced compared with Tr1 cells generated with COX-2⁺ HNSCC cells (Fig. 4D).

COX-2 gene transfection into HNSCC cell line. The HNSCC tumor cell line negative for COX-2 expression (ANT-1) was transfected either with cDNA encoding COX-2 or with an empty vector (pcDNA3.1) as control. Successful transfection was confirmed by immunoblotting for COX-2 protein expression (Fig. 5A), RT-PCR (not shown), and the presence of COX-2 metabolite PGE₂ in the supernatants (Fig. 5B). T lymphocytes from cocultures with tumor cells transfected with the COX-2 gene had significantly higher suppressor activity compared with T lymphocytes cocultured with COX-2-negative cells (Fig. 5C). Cytokines used in the Tr1 IVA had no effect on COX-2 transfected tumor cells (data not shown). Also, the phenotype of T cells outgrowing in cocultures containing COX-2⁻ (parental) versus COX-2⁺ (transfected) tumor was distinct as reported above for siRNA experiments (data not shown).

Levels of cytokines in culture supernatant. The supernatants of different Tr1 cultures were analyzed for levels of cytokines and growth factors considered to be relevant to suppressor function of Tr1 cells. Levels of Tr1 cytokines (IL-10 and TGF-β₁), Th1 cytokines (IFN-γ, TNF-α, IL-2, and IL-12), and Th2 cytokines (IL-4, IL-5, IL-6, IL-8, and IL-10) were correlated with the type of suppressor cell that was induced and outgrew in the IVA cultures. Supernatants of all Tr1 cultures, including IVA cultures containing COX-2⁺ tumor cells, were positive for IL-10, TGF-β₁, and PGE₂ (Table 1) and showed low levels or no IL-2, IL-4, and IFN-γ (data not shown). However, supernatants of COX-2⁻ Tr1 cultures or those with COX-2⁺ tumor cells treated with the COX inhibitor had significantly lower ($P < 0.0001$) levels of IL-10, TGF-β₁, and PGE₂ than supernatants of COX-2⁺ Tr1 cultures. Levels of these cytokines were comparable in supernatants of cultures containing either

Table 1. Levels of cytokines measured in culture supernatant of IVA cultures

	IL-10	<i>P</i>	TGF-β ₁	<i>P</i>	PGE ₂	<i>P</i>
Activated T cells	7 ± 4	}0.0001	88 ± 15	}0.0001	14 ± 4	}0.0002
COX-2 ^{neg} IVA	370 ± 49		443 ± 33		51 ± 8	
COX-2 ^{pos} IVA	615 ± 15	}0.03	824 ± 22	}0.17	98 ± 12	}0.10
PGE ₂ IVA	559 ± 34		846 ± 27		86 ± 6	
COX-2 ^{pos} IVA + Diclofenac	272 ± 8	}0.0001	297 ± 9	}0.0001	49 ± 6	}0.002

NOTE: Supernatants from different IVA cultures were collected on day 10 as described in Materials and Methods and tested for cytokine levels by EIA. Results are mean ± SD cytokine levels (in pg/mL) measured in supernatants of different IVA cultures done with cells obtained from 10 different normal donors.

COX-2⁺ tumor cells or PGE₂. In addition, supernatants of all Tr1 cultures were negative for IL-1 β , IL-2, IL-5, IL-6, IL-8, IL-12, IFN- γ , TNF- α , GM-CSF, and nitric oxide (data not shown). Supernatants from reference cultures (activated CD4⁺ T cells) were very low or negative for IL-10 and TGF- β 1 and contained low to intermediate levels of some of the other cytokines tested (e.g., IL-2, IL-4, and IFN- γ ; data not shown). Supernatants from all cultures of tumor cells alone were negative for the cytokines in the panel, including TGF- β 1 and nitric oxide, but were positive for PGE₂ (Figs. 4B and 5B).

Discussion

Overexpression of COX-2 at sites of chronic inflammation and in human malignancies is accompanied by imbalance and suppression of the immune system. HNSCC is highly immunosuppressive, and overexpression of COX-2 is a common feature of this type of cancer (7). It has been known for many years that tumors produce a variety of immunosuppressive factors, which affect functions of immune cells not only *in situ* but also systemically (23–26), and COX-2 production has been considered to be a negative prognostic factor in HNSCC (27).

In this study, we focused on effects of COX-2 overexpression in tumor cells on the regulatory T-cell subset Tr1. Using the recently established Tr1 IVA system (19) and HNSCC cell lines which constitutively express COX-2 or not, it was possible to show that an active COX-2 pathway in tumor cells promotes generation of Tr1 cells, which produce immunosuppressive cytokines IL-10 and TGF- β and mediate strong suppressive activity. Interference with COX-2 expression by siRNA in COX-2-positive tumor cells significantly reduced outgrowth of Tr1 cells and their suppressor activity. In contrast, the COX-2 gene transfection into tumor cells negative for the enzyme-facilitated outgrowth of Tr1 cells is capable of suppression. Our results indicate that COX-2 and PGE₂ are, at least in part, responsible for the generation of Tr1 suppressor cells and for creating a tolerogenic microenvironment *in situ* as previously suggested (11). In aggregate, these findings provide additional evidence for the central role of COX-2 and PGE₂ in immunosuppression, which is associated with HNSCC progression.

Recently, evidence has emerged, suggesting that tumor-derived PGE₂ is involved in promoting expression of suppression-related transcription factor FoxP3 in CD4⁺CD25⁺ regulatory T cells (5, 10). Furthermore, it has been reported that COX-2 is expressed in CD4⁺CD25⁺ adaptive Tregs and might be responsible for FoxP3⁺ up-regulation and suppression of effector T-cell responses (28). In our IVA model, we have also observed a significant up-regulation of FoxP3 expression in Tr1 cells promoted by a COX-2⁺ tumor environment, but not by synthetic PGE₂ (data not shown). This suggests that factors other than PGE₂ present in the coculture of tumor cells with lymphocytes (e.g., tumor antigens) may be critical for up-regulation of this suppression-related molecule. On the other hand, synthetic PGE₂ in Tr1 IVA significantly promoted outgrowth of cells positive for IL-2R γ , a receptor which may be important for Tr1 cell functions. A cross-link of the PGE₂ signaling pathway with pathways triggered by cytokines which use the common γ chain might be implicated in Tr1 cell activation.

Akasaki et al. described an *in vitro* model, in which a COX-2-overexpressing glioma cell line induced IL-10-secretion in matured dendritic cells, resulting in a Tr1 cell response (11). Stolina et al. reported tumor COX-2 expression to be a pivotal determinant of IL-10 expression and, hence, also of tumor-mediated immune suppression (29). Thus, the differentiation of Tr1 cells is also

controlled by dendritic cell subsets, which can produce IL-10 and may express tolerogenic costimulatory molecules (30). Moreover, PGE₂ has been reported to enhance IL-10 production, down-regulate APC function and inhibit IL-12 production in dendritic cell (31). We also detected no IL-12 in Tr1 IVA while culturing iDC alone with Tr1 cytokines or PGE₂ (data not shown). Thus, PGE₂ production by the tumor may lead to induction of Tr1 cells in naive CD4⁺CD25⁻ T-cell populations (32) and contribute to creating a tolerogenic microenvironment.

The origin of Tr1 cells, which outgrow in the presence of COX-2-overexpressing tumor cells, remains undefined. On the one hand, it is possible that Tr1 cells are derived from naive CD4⁺CD25⁻ T cells, which migrate to the tumor microenvironment and are converted under the PGE₂ influence into regulatory T cells. On the other hand, Tr1 cells might represent nTreg (CD4⁺CD25⁺) which are enriched in the tumor and are converted through influence of the COX-2 pathway into a subset of suppressor cells with a distinct phenotype and a characteristic mechanism of suppression. It is clear that in the tumor microenvironment, different subtypes of regulatory T cells exist, which are shaped to promote tumor growth and down-regulate host immune responses. In this process, dendritic cells may act as an intermediary and relay tumor-derived signaling to responding lymphocytes in a primarily IL-10-dependent way. An increasing number of reports suggest that PGE₂ acts in concert with IL-10 in inducing immune tolerance in cancer (33). Hence, aggressive, PGE₂-producing tumors, such as many HNSCC, use Tr1 cells to successfully evade the host immune system in a PGE₂-dependent way. In the tumor microenvironment, Tr1 cells are induced to secrete IL-10 and TGF- β , which suppress T-cell responses. IL-10 can inhibit T-cell functions, both indirectly through its down-regulation of APC functions and directly through inhibition of cytokine production (34). Hence, suppression mediated by Tr1 cells is cytokine-dependent and cell contact-independent (22), as confirmed in our blocking experiments with neutralizing Abs and transwell inserts. This mechanism of suppression is another classic feature of Tr1 cells that distinguishes them from nTreg.

The concept of immunorestorative strategies via COX inhibition in cancer is supported by multiple studies (12, 35, 36). The COX inhibitor Diclofenac, which was used in our study, is a member of the group of nonsteroidal antiinflammatory drugs. Diclofenac also inhibits the lipooxygenase pathways, thus reducing formation of leukotrienes, which are proinflammatory autacoids (37). Blocking of the COX-2 pathway with this agent *in vitro* significantly impaired Tr1-cell generation. It is reasonable to predict that the process of carcinogenesis could be antagonized *in vitro* and *in vivo* by selective modulation of the activity of specific enzymes, such as COX-2 and, thus, inhibition of Tr1 cell expansion. In this context, we have recently reported that delivery of a COX-2 inhibitor, Rofecoxib, to HNSCC patients resulted in an increase in tumor-infiltrating Th1 and CD25⁺ effector T cells (38), shifting the antitumor immune response from tolerogenic to immunogenic. This suggests that cancer immunotherapy using COX-2 inhibitors is likely not only to prevent induction of Tr1 cells but may also reverse suppressive effects of already preexisting Tr1 cells.

Others reported that suppression of COX-2 in tumor cells is sufficient to enhance their sensitivity to chemotherapy (39). Furthermore, COX-2 inhibition triggers expression of several death receptors, engaging different apoptosis pathways in cancer cells (40). Molecular mechanisms involved in modulating the COX-2 pathway in tumor cells and its effects on immune suppression have

been extensively investigated. In mice, COX-2 induces arginase I expression in myeloid suppressor cells (41). It would be of considerable interest to determine whether myeloid suppressor cells accumulating in the tumor microenvironment induce Tr1 cell expansion or activity and whether tumor-derived PGE₂ induces L-arginase or indoleamine-2,3-dioxygenase expression in Tr1 cells as yet another tumor-induced mechanism of immune suppression. COX-2 also impairs cellular immunity through cyclic AMP-dependent mechanisms (42). In addition, tumor-derived COX-2 cross-talks with tyrosine kinases associated with epidermal growth factor receptor and vascular epidermal growth factor receptor via direct transactivation (43, 44). Altogether, COX-2 seems to be a particularly relevant target for blocking tumor escape mechanisms.

The evidence we provide that tumor cells, which overexpress COX-2 and secrete PGE₂, can use the Tr1 subset of suppressor cells to counteract antitumor immune responses supports the strategy of combining COX-2 inhibitors with conventional oncologic therapies for treatment of cancer.

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