

Prevention of Both Direct and Cross-Priming of Antitumor CD8⁺ T-Cell Responses following Overproduction of Prostaglandin E₂ by Tumor Cells *In vivo*

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Abstract

Defects in antitumor immune responses have been associated with increased release of prostaglandin E₂ (PGE₂) as a result of overexpression of cyclooxygenase (COX)-2 by tumors. In this report, we examine the effects of PGE₂ on antitumor CD8⁺ T-cell responses generated both by cross-presenting dendritic cells and by direct priming by tumor cells. Our data show that PGE₂ inhibits dendritic cell maturation, resulting in the abortive activation of naïve CD8⁺ T cells, and is dependent on interleukin-10 production by dendritic cells. Interaction of tumor cells with naïve CD8⁺ T cells in the presence of PGE₂ *in vitro* results in the induction of CD8⁺ CD28⁻ T cells, which fail to proliferate or exhibit effector function. *In vivo*, overexpression of COX-2 by tumor cells results in a decrease in number of tumor-infiltrating dendritic cells and confers the ability of tumor cells to metastasize to the tumor draining lymph nodes. [Cancer Res 2008;68(18):7520–9]

Introduction

Activation of naïve tumor-specific CD8⁺ T cells occurs either through direct interaction with metastatic tumors in the tumor draining lymph nodes (TDLN), or indirectly via dendritic cells, cross-presenting tumor-derived antigens to naïve CD8⁺ T cells within the TDLN. Deficiencies in dendritic cell recruitment and antigen-presenting cell (APC) function have been observed in many human cancers, resulting in reduced T-cell proliferation and effector function (1, 2). In some cases, these defects in dendritic cell function were associated with the release of tumor-induced factors, including interleukin (IL)-10 and prostaglandin E₂ (PGE₂; refs. 3–5). IL-10 production can be up-regulated by PGE₂ (6, 7) and has been shown to have anti-inflammatory activity, dampening CD4⁺ and CD8⁺ T-cell responses by inhibiting maturation (8), and cytokine release (9) by dendritic cells resulting in T-cell anergy (2, 10). Overexpression of PGE₂ has been shown in many cancers (5, 11), causing reduced number of tumor-infiltrating dendritic cells and a reduction in their APC function (12, 13). PGE₂ has been shown to shift the profile from antitumor Th1 responses to Th2 responses, characterized by reduced IL-12 and increased IL-10 secretion by dendritic cells (14). Furthermore, PGE₂ increases tumor cell survival (15), motility (16), and angiogenesis (17), thus allowing them to metastasize to the TDLN. Although metastasis provides a means of direct CD8⁺ T-cell activation (18), migration of

COX-2-overexpressing tumors to the TDLN fails to induce antitumor T-cell responses (19).

To investigate the effects of PGE₂ on antitumor CD8⁺ T-cell responses, we have adapted a murine renal cell carcinoma (Renca) model in which the hemagglutinin antigen (HA) of influenza virus A/PR8 H1N1 is expressed as a neo-antigen (20). RencaHA cells were transfected with murine COX-2 cDNA (T3), resulting in the overexpression of COX-2 and PGE₂. CD8⁺ T-cell responses to these tumor cell lines were investigated using transgenic CL4 CD8⁺ T cells expressing TCRs with high affinity for the immunodominant K^bHA-restricted epitope (21). Our data clearly show that PGE₂ abrogates antitumor specific CTL responses *in vivo* and *in vitro* by down-regulating both direct antigen presentation and cross-presentation pathways.

Materials and Methods

Mice. Six- to 8-wk-old BALB/c and BALB/c CL4 TCR transgenic mice (21) were bred and maintained under specific pathogen-free conditions at the University of Bristol Animal Services Unit. All experiments were conducted in accordance with the U.K. Home Office guidelines.

Cell lines. The RencaNT cell line was single cell cloned from a population of Renca cells (22). The RencaHA (20) COX-2 (T3) cell line was established by transfecting the RencaHA cell line. Briefly, a full-length COX-2 cDNA PCR product containing flanking *NotI* restriction sites was generated using the following primers: 5'-ATAAGAATGCGGCCGATGCTCTTCC-GAGCTGTGC-3' and 5'-ATAGTTTAGCGGCCGCTCATTACAGCTCAGTT-GAACGCC (the source plasmid was kindly provided by Prof. R. Dubois, Vanderbilt University Medical Center and Vanderbilt-Ingram Cancer Center, Nashville, TN), and cloned into the *NotI* restriction site of the expression vector pIRESpuo2 (Clontech). RencaHA cells were plated in six-well plates before transfection. When 70% to 90% confluent, cells were incubated with 10 µg of Midiprep DNA (Qiagen) mixed with Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. Cells were then incubated in the absence of antibiotics penicillin/streptomycin (Invitrogen) and FCS (Invitrogen) for 72 h, after which cells were washed with HBSS (Invitrogen), and grown in medium supplemented with 1 µg/mL puromycin (Sigma-Aldrich) and 0.1 mg/mL geneticin (Invitrogen). Resulting colonies were expanded and assayed for integrated COX-2 cDNA by PCR and COX-2 mRNA expression by reverse transcription-PCR (RT-PCR) and for PGE₂ production by ELISA. RencaNT cells were maintained in complete medium [RPMI 1640 (Sigma-Aldrich) supplemented with 10% (v/v) FCS, 2 mmol/L glutamine, 50 units/mL penicillin/streptomycin, and 5 × 10⁻⁵ mol/L 2-ME, all obtained from Invitrogen]. RencaHA cells were grown in complete medium supplemented with 0.1 mg/mL geneticin (Invitrogen), and T3 were maintained in complete medium containing 0.1 mg/mL geneticin and 1 µg/mL of puromycin (Sigma-Aldrich).

Bone marrow-derived dendritic cells. Bone marrow-derived dendritic cells (BMDC) were generated from hematopoietic progenitor cells (23). Briefly, bone marrow cells were harvested aseptically from the leg bones of BALB/c mice by flushing out the lumen of the bones with complete RPMI using a 25-gauge needle (Tyco Healthcare), in plastic Petri dishes. Cells were filtered through a 40-µm cell strainer. Macrophages were depleted after

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3-h incubation of bone marrow cells in granulocyte macrophage colony-stimulating factor (GM-CSF) medium [complete RPMI containing 10% (v/v) GM-CSF obtained from supernatant of GM-CSF-secreting X63 hybridoma cell line, a gift from Dr. F. Ronchese, Malaghan Institute of Medical Research, Wellington University School of Medicine, Wellington, New Zealand] in T75 flask at 37°C in a humidified incubator with 5% (v/v) CO₂. After 3-h incubation, the supernatant was centrifuged at 1,800 rpm for 5 min and the cells were resuspended at 2×10^6 /mL. Two million cells per well were cultured in 6-well plates in GM-CSF medium, for 10 d at 37°C in a humidified incubator. The medium was changed on days 3, 6, and 7 by replacing 2 mL of medium with fresh GM-CSF medium. Matured dendritic cells (mDC) were generated by the addition of lipopolysaccharide (LPS; 1 µg/mL; *Escherichia coli* 026:B6, Sigma-Aldrich) to the immature dendritic cell (iDC) culture for 18 h. Where mentioned on day 7 or 9, iDC were incubated with 1 µmol/L PGE₂ (Sigma-Aldrich) followed by 1 µg/mL LPS (Sigma-Aldrich) on day 9. On day 10, nonadherent cells were harvested from the wells, washed thoroughly, irradiated with 3,000 rad, and used as APC, as described below. Where mentioned, dendritic cells were treated with 15 µg/mL of purified anti-IL-10R monoclonal antibody (mAb; 1B1.3 clone; ref. 24) or normal rat IgG isotype (Invitrogen) on day 9.

ELISA. The concentration of IL-10 in the supernatant of BMDC was measured using standard ELISA protocols. Plates were coated with purified anti-IL-10 mAb (Biosource Oxford Biosystems), blocked with 1% bovine serum albumin in PBS, and incubated with supernatants for 2 h, followed by biotinylated anti-IL-10 mAb (Biosource). Bound biotinylated mAb was detected by incubation with ExtrAvidin-HRP (Sigma-Aldrich) followed by incubation with tetramethylbenzidine substrate (Sigma-Aldrich). The reaction was stopped with 2 mol/L H₂SO₄. Absorbance was read at 450 nm with a 595 nm reference. Supernatant from RencaHA and T3 tumor cell lines were analyzed for PGE₂ by ELISA, using the PGE₂ EIA kit (Assay Designs Cambridge) according to the manufacturer's instructions. Briefly, tumor cells left untreated or treated with 5 µg/mL NS-398 [Sigma-Aldrich; dissolved in 10% (v/v) DMSO] for 72 h were pretreated with 44 µmol/L arachidonic acid (Sigma-Aldrich) for 45 min before the supernatant was collected and frozen at -80°C. The number of cells for each tumor was counted and the concentration of PGE₂ obtained from ELISA was normalized to 1×10^6 cells.

Enrichment of CL4 CD8⁺ T cells. Single-cell suspensions were generated from CL4 TCR transgenic mice by positive selection using anti-CD8 MACS beads on midiMACS (Miltenyi Biotec Bisely) and LS separation columns, according to the manufacturer's instructions. This routinely gave a purity of CD8⁺ T cells >95% as determined by flow cytometry. In some experiments, CL4 CD8⁺ T cells were labeled with 5 µmol/L CFSE (Molecular Probes, Inc.) in accordance with described protocols (23).

In vivo proliferation assays. BALB/c mice were injected s.c. with 1×10^6 RencaHA or T3 on day 0. On day 11, mice were injected i.v. with 3×10^6 purified, naïve CFSE-labeled CL4 CD8⁺ T cells. For COX-2 inhibition, mice were injected i.p. with 10 mg/kg NS-398 (Sigma-Aldrich) dissolved in 10% (v/v) DMSO. The drug was administered before tumor injection and then thrice per week for the duration of the experiment. Tumor growth was determined on days 16 and 21 (20). Tumors were measured with calipers and the growth was evaluated by applying the following formula ($a^2 \times b/2$), where a is the horizontal diameter and b is the vertical diameter of the tumor. For assessment of CL4 accumulation in the TDLN and tumor, mice were sacrificed on days 16 and 21, respectively.

Coculture and CL4 CD8⁺ T-cell proliferation in vitro. CL4 CD8⁺ T-cell proliferation assays were performed as described previously (23). Briefly, 1×10^4 naïve CL4 CD8⁺ T cells were cocultured with 1×10^4 irradiated BMDC in 96-well plates in the presence of 1 ng/mL K^dHA peptide (IYSTVASSL), at 37°C, 5% (v/v) CO₂ for 72 h. For the final 8 h, cells were pulsed with 1 µCi/well [³H]thymidine (Amersham Life Sciences Buckinghamshire). [³H]thymidine incorporation was measured using a 1450 Microbeta liquid scintillation counter with Microbeta for Windows 2.7 (Wallac-Oy). For coculture assays, 1×10^6 naïve CFSE-labeled CL4 CD8⁺ T cells were cultured with 1×10^5 irradiated BMDC, splenocytes, or tumors in 24-well plates. For experiments using BMDC and splenocytes, 1 ng/mL K^dHA (IYSTVASSL) peptide was added to the culture at the time of

incubation with CL4 CD8⁺ T cells. After 48 to 72 h at 37°C, 5% (v/v) CO₂, CL4 CD8⁺ T cells were examined by flow cytometry for intracellular IFN-γ or for the expression of cell surface molecules. For IL-10R blocking experiments, CL4 CD8⁺ T cells were cocultured with dendritic cells in the presence of 15 µg/mL anti-IL-10R mAb (24) or rat IgG1 isotype (Invitrogen) for 72 h.

Flow cytometry. Single-cell suspensions derived from BMDC were stained with anti-CD11c.PE mAb in the presence of supernatant from anti-FcγIII mAb-secreting 2.4G2 cell line. CD11c⁺ cells were analyzed for cell surface molecules using anti-H2K^d.bio, CD80.bio, CD86.bio, CD40.bio mAbs, or isotype controls followed by SA.APC. CL4 CD8⁺ T cells were stained by anti-Thy1.1.PE, CD69.bio, CD28.bio, and Ly6C.bio followed by SA.APC. All these mAbs were purchased from BD Oxford. Dead cells were stained with 7-amino-actinomycin D (Sigma-Aldrich) and gated out in the analyses. Intracellular IFN-γ and CTLA-4 expression in CL4 CD8⁺ T cells were detected using the BD Perm/fix kit with Golgi plug (BD) according to the manufacturer's instructions and stained with anti-IFN-γ.APC mAb or anti-CTLA-4.PE (BD). The expression of all surface and intracellular molecules except of CTLA-4 was measured after 72 h culture. Expression of the intracellular CTLA-4 in CL4 CD8⁺ T cells was measured after 48 h culture. RencaHA, T3, and RencaNT cell lines were stained for HA, using 37/38 mAb followed by goat anti-mouse IgG-FITC (Sigma-Aldrich) secondary antibody. MHC class I expression in RencaHA and T3 cell lines was detected by anti-H2K^d.bio mAb followed by SA.APC. Cells were acquired on a FACSCalibur flow cytometer with CellQuest software (BD Cytometry Systems Oxford).

PCR and RT-PCR. For HA RT-PCR, TDLN from mice with T3 or RencaHA were isolated and total RNA was extracted from single-cell suspension using TRIzol reagent (Invitrogen) following manufacturer's instructions (20). cDNA was synthesized using a cDNA synthesis kit (Invitrogen). For the HA PCR, the following primers were used: 5'-CAATTGGGGAAATGTAACATCGCCG-3', 5'-AGCTTTGGGTATGAGCCCTCCTTC-3'; cycling conditions were 94°C/5 min, 28 cycles for 94°C/30 s, 61°C/30 s (HA). The stable integration of the COX-2 cDNA into the T3 genome was examined by PCR. COX-2 RT-PCR was performed on single-cell suspensions of RencaHA and T3 cell lines from *in vitro* cultures using the RETROscript reverse transcription kit (Ambion), according to the manufacturer's instructions. The COX-2 primer sequences used were 5'-ATGCTCTCCGAGCTGTGC-3' and 5'-TTA-CAGCTCAGTTGAACGCC-3'. The hypoxanthine phosphoribosyltransferase (HPRT) primers used were as follows: 5'-GTTGGATACAGCCA-GACTTTGTTG-3' and 5'-GAAGGTTAGGCTGGCCATAGGCT-3'. For COX-2, the PCR cycle conditions were 94°C/2 min and 30 s, 94°C/30 s, 57°C/30 s, 72°C/30 s for 30 cycles and finally 72°C/7 min. For HPRT, the PCR cycle conditions were 94°C/2 min and 30 s, 94°C/30 s, 60°C/30 s, 72°C/30 s for 28 cycles and finally 72°C/7 min.

Statistical analysis. *P* values were calculated with the Student's *t* test using the Prism 4.03 software (GraphPad Software, Inc.), with two-tailed distribution and two-sample equal variance parameter.

Results

PGE₂ inhibits dendritic cell maturation in vitro. Previous studies *in vitro* have shown that PGE₂ exerts different effects on the cytokine profile of dendritic cells depending on their stage of differentiation and maturation (25). We wished to determine whether dendritic cell maturation was altered if PGE₂ was present during differentiation. iDC were generated *in vitro* from bone marrow stem cells in the presence or absence of exogenous PGE₂, either before or during LPS maturation (Fig. 1A). Treatment of iDC cultures with LPS on day 9 of differentiation resulted in dendritic cell maturation to form mDC, as evidenced by increased expression of MHC class I, CD80, CD86, and CD40, compared with iDC. However, when iDC were differentiated in the presence of PGE₂, expression of MHC class I was reduced compared with untreated iDC, yet up-regulation of CD80 and CD86 was observed. Importantly, treatment of iDC with PGE₂ at the same time as

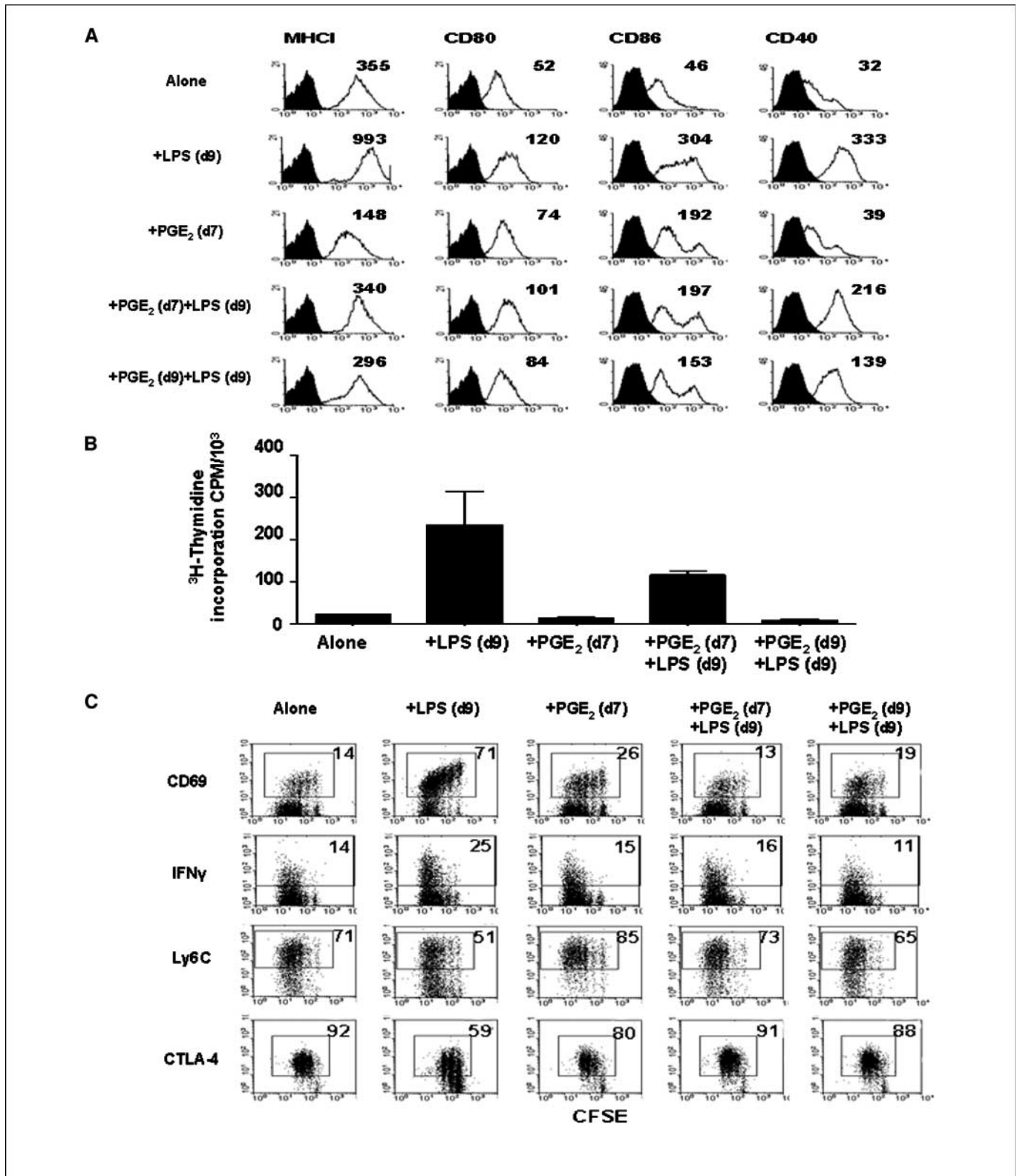


Figure 1. PGE₂-treated dendritic cells induce abortive activation of CL4 cells *in vitro*. **A**, iDC were either left untreated (*Alone*), treated with 1 μ g/mL LPS for 18 h on day 9 [*+LPS(d9)*], treated with PGE₂ (10⁻⁶ mol/L) alone on day 7 [*+PGE₂(d7)*], or treated with PGE₂ (10⁻⁶ mol/L) on day 7 followed by LPS on day 9 [*+PGE₂(d7)+LPS(d9)*]. Cells were alternatively treated with a mixture of PGE₂-LPS on day 9 [*+PGE₂(d9)+LPS(d9)*]. Cells harvested on day 10 were analyzed by flow cytometry for cell surface receptor expression as indicated in the figure (*open histograms*). *Filled histograms*, isotype controls. The data are from one of eight performed experiments. Number in each histogram represents the mean fluorescence intensity. **B**, CL4 proliferation as measured by [³H]thymidine incorporation after coculture with iDC treated as described in **A**. *Columns*, mean from one of three performed experiments, each with triplicates; *bars*, SD. **C**, phenotypic analyses of Thy1.1⁺ CFSE-labeled CL4 cells after coculture with iDC treated as described in **A**. Numbers indicate the percentage of divided CL4 cells expressing the receptors as indicated in the figure. The data are from one of two performed experiments.

LPS prevented increased expression of MHC class I, CD80, CD86, and CD40 compared with untreated LPS-mDC. Similarly, the presence of PGE₂ during differentiation into iDC reduced their ability to mature. However, CD40 and CD86 expression was higher among dendritic cells differentiated in the presence of PGE₂, and matured with LPS, compared with dendritic cells matured in the presence of PGE₂. Taken together, these data suggest that PGE₂ renders iDC refractory to maturation signals when present either during differentiation, or during maturation, with the latter being more susceptible to the effects of PGE₂.

PGE₂ present during dendritic cell maturation abrogates CL4 T-cell activation *in vitro*. To evaluate the consequences following priming of naïve CD8⁺ T cells by PGE₂-conditioned dendritic cells, purified naïve CL4 cells were cocultured with PGE₂-treated, K^dHA peptide-pulsed dendritic cells, and their ability to proliferate and exhibit CTL effector function was analyzed *in vitro* (Fig. 1B and C). Coculturing naïve CL4 cells with mDC resulted in a 10-fold increase in proliferation, compared with co-culturing in the presence of iDC. PGE₂ treatment of iDC did not affect proliferation of cells compared with untreated iDC. However, coculturing CL4 cells with LPS-mDC treated with PGE₂ during their differentiation reduced CL4 proliferation compared with CL4 cells cocultured with LPS-mDC. Critically, treatment of iDC with PGE₂ at the same time as LPS abrogated CL4 cell proliferation. These data suggest that conditioning of dendritic cells with PGE₂ suppresses their APC function, which is greatest during dendritic cell maturation.

Following naïve CL4 cell coculture with iDC, few CL4 cells increased expression of CD69 and elaborated IFN- γ upon restimulation with K^dHA peptide. Furthermore, >70% and 90% of CL4 cells were Ly6C^{hi} and CTLA-4^{hi}, respectively (Fig. 1C). Such a phenotype is consistent with the cell surface phenotype of CL4 CD8⁺ T cells undergoing abortive activation and peripheral tolerance induction (23, 26, 27). When naïve CL4 cells were cocultured with LPS-mDC, >70% of CL4 cells expressed CD69, the number of IFN- γ ⁺ cells increased, and only around half of the cells were Ly6C^{hi} and CTLA-4^{hi}, suggesting that these cells were undergoing productive activation to form effector CTL (23). Following PGE₂ treatment, iDC were still able to induce abortive activation of CL4 cells; however, PGE₂ treatment prevented productive activation by mDC. The shift in response in favor of abortive activation occurred regardless of whether dendritic cells were treated with PGE₂ during differentiation or maturation.

Blocking IL-10R *in vitro* reverses the inhibitory effect of PGE₂ on dendritic cell function. Thus far, the data suggest that PGE₂ exerts immunosuppressive effects on dendritic cell function. PGE₂ is known to cause increased production of IL-10 by dendritic cells (28), which has been shown to reduce dendritic cell maturation (8, 29), and dampen productive activation of CD8⁺ T cells by dendritic cells (30). Therefore, we wished to evaluate the levels of IL-10 production by dendritic cells during differentiation and LPS maturation in the presence or absence of PGE₂ by ELISA (Fig. 2A). IL-10 production was approximately equal among both iDC and LPS-mDC. Treatment of iDC with PGE₂ alone had no effect on IL-10 production compared with either untreated iDC or with mDC (Fig. 2A). However, when PGE₂ was present during dendritic cell differentiation followed by LPS maturation, IL-10 production increased by 2-fold compared with LPS-mDC. Moreover, when PGE₂ was present during LPS maturation on day 9, IL-10 production increased by >3-fold compared with dendritic cells matured without PGE₂.

To investigate whether the production of IL-10 by dendritic cells has a role in abortive activation, iDC were treated with anti-IL-10R mAb during maturation with LPS in the presence or absence of PGE₂, and used as APC for CL4 CD8⁺ T-cell activation *in vitro*. Subsequently, CL4 cells isolated from the cultures were analyzed for IFN- γ production. Treatment of iDC and mDC with the anti-IL-10R mAb resulted in an increase in IFN- γ ⁺ CL4 cells compared with those cocultured with isotype-treated iDC and mDC, respectively (Fig. 2B). Similarly, anti-IL-10R mAb-treated iDC, which were conditioned with PGE₂ during maturation, significantly increased the %IFN- γ ⁺ CL4 cells to the same level as the isotype-treated mDC cultures. To examine whether blocking IL-10 during dendritic cell-CL4 cell interaction enhances CL4 effector function, CL4 cells were cultured with dendritic cells in the presence of the anti-IL-10R mAb or an isotype control mAb, and then analyzed for IFN- γ production. Figure 2C shows that treatment of mDC-CL4 cell cocultures with anti-IL-10R mAb did not alter the proportion of IFN- γ ⁺ CL4 cells compared with isotype-treated mDC cocultures. However, blocking of IL-10R in iDC cocultures resulted in a significant increase in %IFN- γ ⁺ CL4 cells above that of both isotype-treated and anti-IL-10R mAb-treated mDC cocultures. Regardless of the presence or absence of anti-IL-10R mAb, coculturing of naïve CL4 with iDC conditioned with PGE₂ during maturation did not result in an increase in proportion of IFN- γ ⁺ CL4 cells compared with coculturing with mDC (Fig. 2C).

COX-2-overexpressing RencaHA (T3) tumor cells do not prime naïve CL4 cells *in vivo*. Previous studies in our laboratory have shown that cross-presentation of RencaHA cell-derived K^dHA epitopes by BMDC results in priming of naïve K^dHA-specific CL4 CD8⁺ T cells (21) within the TDLN of conventional BALB/c mice, giving rise to the formation of CTL effector and tumor-infiltrating T cells (TIL; ref. 20). To examine the effects of COX-2 overexpression on the induction of tumor-specific CTL responses *in vivo*, RencaHA cells were transfected with a murine COX-2 cDNA to establish a COX-2-overexpressing cell line (T3; Fig. 3A and B), which resulted in a significant increase in PGE₂ release by T3 cells *in vitro* (Fig. 3C). Importantly, treatment of T3 cells with the COX-2-specific inhibitor NS-398 decreased PGE₂ production by T3 to the same level as that produced by RencaHA, indicating that increased PGE₂ production by T3 was due to the overexpression of COX-2. Importantly, flow cytometric analyses showed that there were equivalent levels of cell surface HA protein and MHC class I expression among the RencaHA and T3 cell lines (Fig. 3D). In addition, both tumors were able to grow at the same rate in BALB/c mice (data not shown).

To examine the effect of COX-2 overexpression on antitumor CL4 cell responses, Thy1.2⁺ BALB/c mice were injected s.c. with 1×10^6 RencaHA or T3 cells on day 0 followed by CFSE-labeled naïve Thy1.1⁺ CL4 cells on day 11. On days 16 and 21, TDLN and tumors were removed, respectively, and CL4 cell infiltration was assessed by flow cytometric analyses (Fig. 4). In mice given RencaHA cells, CFSE-labeled Thy1.1⁺ CL4 cells proliferated in the TDLN, elaborated IFN- γ upon restimulation with K^dHA peptide *in vitro*, and formed TIL. However, despite both T3 and RencaHA tumors growing to a similar size *in vivo*, few Thy1.1⁺ CL4 cells proliferated and produced IFN- γ in the TDLN of T3 tumor-bearing mice, and, as a consequence, very few CL4 cells were present among all of the TIL isolated from the tumors. The lack of CL4 cell proliferation in the TDLN was dependent on COX-2 overexpression, as the use of NS-398 restored CL4 cell proliferation in the TDLN of T3

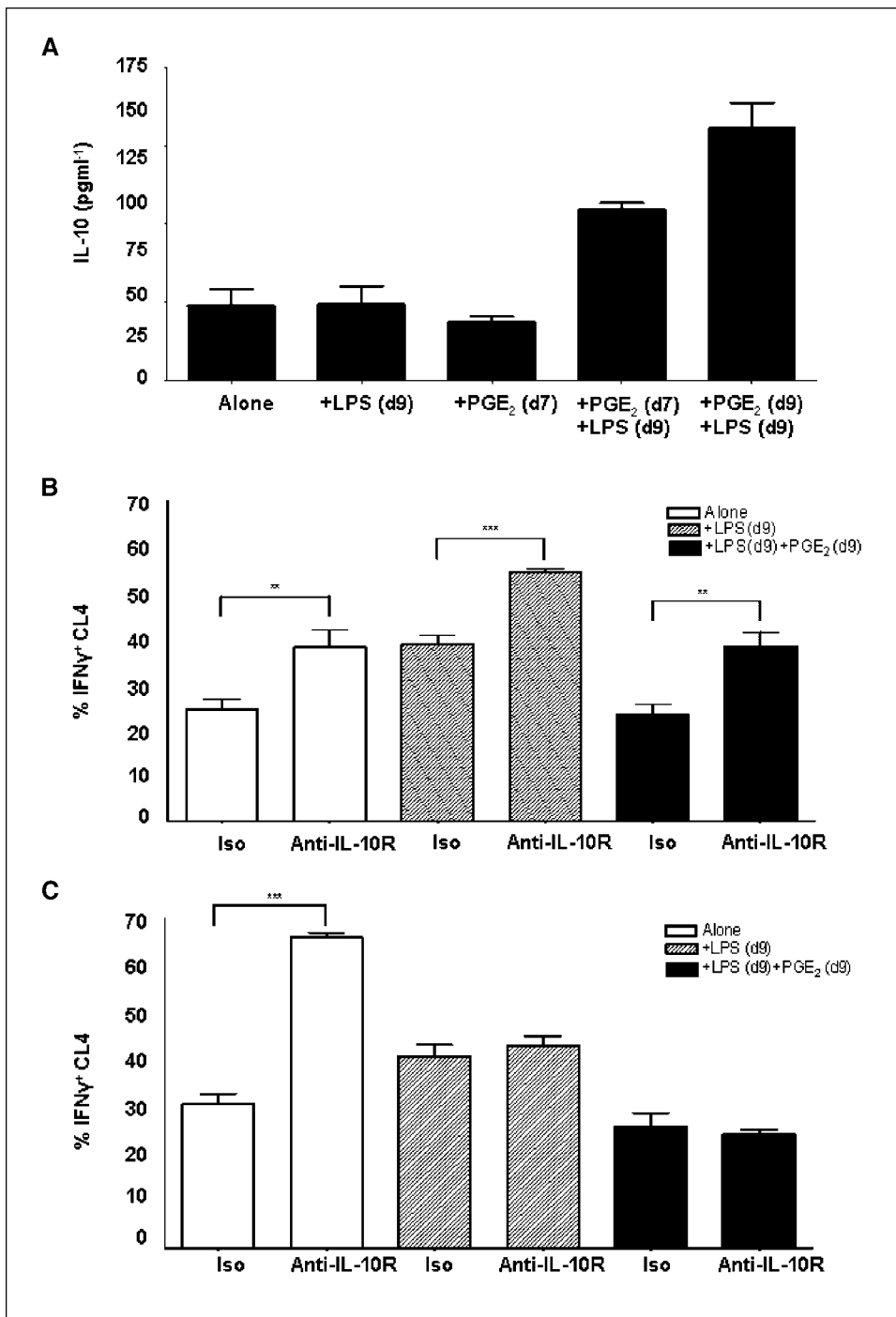


Figure 2. Blocking IL-10 *in vitro* reverses the inhibitory effects of PGE₂ on dendritic cell function. **A**, iDC were either left untreated (*Alone*), treated with 1 μg/mL LPS for 18 h on day 9 [*+LPS(d9)*], treated with PGE₂ (10⁻⁶ mol/L) alone on day 7 [*+PGE₂(d7)*], or treated with PGE₂ (10⁻⁶ mol/L) on day 7 followed by LPS on day 9 [*+PGE₂(d7)+LPS(d9)*]. Cells were alternatively treated with a mixture of PGE₂-LPS on day 9 [*+PGE₂(d9)+LPS(d9)*]. Histogram represents IL-10 production by dendritic cells treated as indicated in the figure. *Columns*, mean from one of two performed experiments, each with triplicates; *bars*, SD. **B**, iDC left untreated (*Alone*), treated with LPS alone on day 9 [*+LPS(d9)*], or PGE₂ and LPS on day 9 [*+PGE₂(d9)+LPS(d9)*] were further treated with purified anti-IL-10R mAb or an isotype control (*Iso*) on day 9, before being washed and cocultured with CFSE-labeled, naïve Thy1.1⁺, CL4 cells. Histograms represent %IFN-γ⁺ CL4 cells isolated from the dendritic cell cocultures, as indicated in the figure. *Columns*, average from two experiments performed in triplicate wells; *bars*, SD. **C**, alternatively, iDC left untreated (*Alone*), treated with LPS alone on day 9 [*+LPS(d9)*], or PGE₂ and LPS on day 9 [*+PGE₂(d9)+LPS(d9)*] were cultured with naïve, CFSE-labeled Thy1.1⁺ CL4 cells and the dendritic cell-CL4 cell cocultures were treated with the anti-IL-10R mAb or the isotype control (*Iso*). Histograms represent %IFN-γ⁺ CL4 cells isolated from dendritic cell cocultures as indicated in the figure. *Columns*, average from two experiments performed in triplicate wells; *bars*, SD. **, *P* between 0.001 and 0.01; ***, *P* < 0.001.

tumor-bearing mice to the same level as that in the TDLN of RencaHA tumor-bearing mice (Table 1). Taken together, these data suggest that COX-2 overexpression by T3 tumors prevents the formation of both tumor-specific CTL and TIL.

CL4 cells undergo abortive activation in response to T3 tumor *in vivo*. To determine whether COX-2 overexpression by T3 induces abortive activation of CL4 CD8⁺ T cells *in vivo*, CFSE-labeled CL4 cells recovered from the TDLN of RencaHA and T3 tumor-bearing mice were characterized for the expression of Ly6C (23). Examination of the TDLN of tumor-bearing mice revealed that, whereas the majority of dividing CL4 cells in the

TDLN of RencaHA tumor-bearing mice were Ly6C^{lo}, the majority of the dividing CL4 cells in the TDLN of T3-bearing mice were Ly6C^{hi} (Fig. 4C). These data suggest that overexpression of PGE₂, due to the presence of the COX-2 transgene, promotes abortive activation and tolerance induction among naïve CL4 cells in the TDLN.

Fewer dendritic cells are present within T3 tumors. Data in Fig. 2 suggest that PGE₂ influences dendritic cell function by promoting IL-10 production. Other studies have shown that IL-10 overexpression by tumor cells reduces accumulation of dendritic cells within the tumor (31). Therefore, flow cytometric analyses of

RencaHA and T3 tumors were carried out to determine the absolute numbers of tumor-infiltrating CD11c⁺ dendritic cells (32). Figure 4D shows a significant increase in the absolute numbers of CD11c⁺ cells within the RencaHA tumors compared with T3 tumors, suggesting that COX-2 overexpression, and increased PGE₂ production that occurs as a result, may influence the accumulation of dendritic cells in the tumor.

Exogenous PGE₂ abrogates direct activation of CL4 cells by tumor cells. The data indicate that PGE₂ overproduction by T3 cells may interfere with cross-presentation by dendritic cells, preventing activation of antitumor CL4 CTL responses *in vivo*. However, other studies have shown that overexpression of COX-2

by tumors increases motility and angiogenesis among tumor cells *in vivo* (16, 17), allowing them to form metastases in the local TDLN. The formation of metastases may provide a mechanism for activation of the antitumor CD8⁺ T-cell responses directly by tumors (18). Therefore, we investigated whether tumor cells were present in the TDLN of BALB/c mice given T3 or RencaHA. On day 16, the TDLN from each group of mice were isolated and cDNA was prepared for RT-PCR analyses. Whereas HA mRNA was not detected in any of the TDLN from RencaHA-bearing mice (Fig. 5), HA mRNA could be detected in the TDLN of T3-bearing mice, suggesting that T3 tumor cells had migrated to these LN.

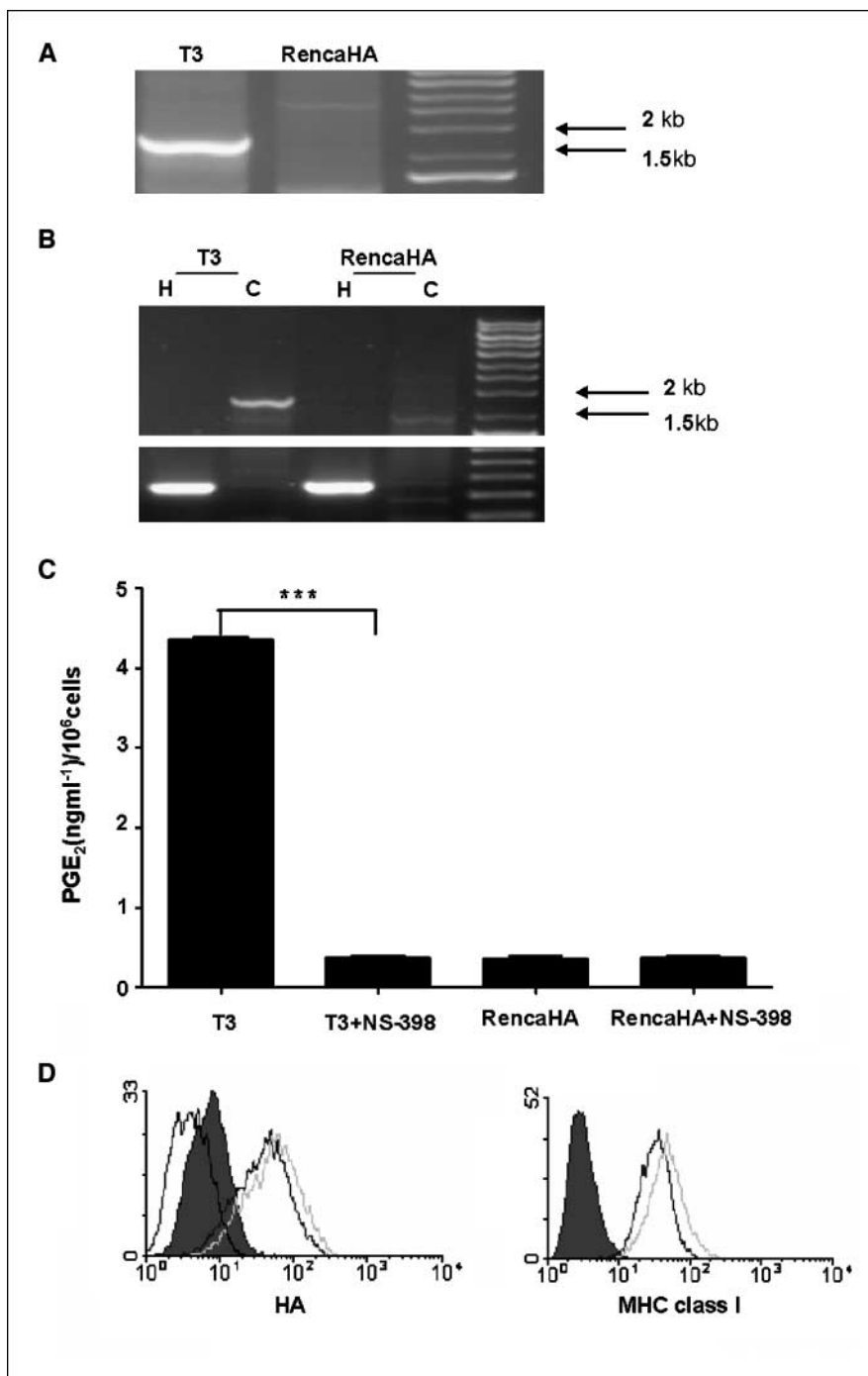


Figure 3. Characterization of RencaHA and T3 cell lines. *A*, PCR, showing the presence of the transfected COX-2 cDNA. *B*, RT-PCR, showing expression of COX-2 by RencaHA and T3 cell lines. *H*, the housekeeping gene *HPRT*; *C*, COX-2. *C*, PGE₂ produced by T3 and RencaHA cell lines *in vitro* left untreated or treated with 5 μg/mL NS-398, as measured by ELISA. *D*, HA and MHC class I expression by T3 and RencaHA was measured by flow cytometry. *Black line*, expression on RencaHA cells; *gray line*, expression on T3 cell line; *dotted line* (HA expression only), expression on RencaNT cell line. *Filled histogram*, control secondary mAb alone. The data are from one of six performed experiments. *******, *P* < 0.001.

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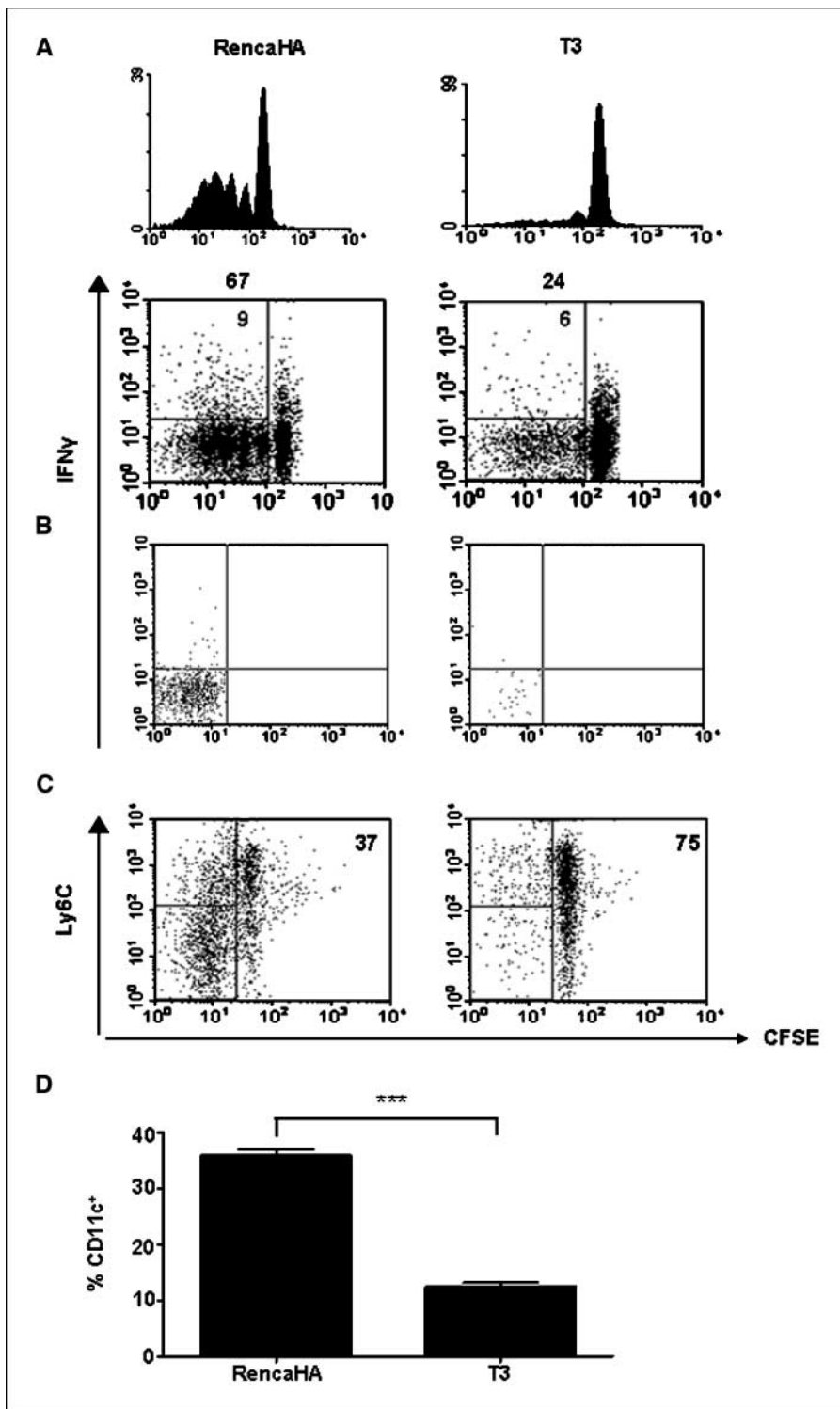


Figure 4. CL4 cells fail to proliferate in response to T3 *in vivo*. Thy1.2⁺ BALB/c mice were injected s.c. with RencaHA or T3 tumor cells. On day 11, mice were given 3×10^6 naïve, CFSE-labeled Thy1.1⁺ CL4 cells i.v.; on day 16 or 21, cells obtained from the TDLN or the tumor, respectively, were stained for Thy1.1. Data represent proliferation and intracellular staining of IFN- γ among CL4 cells isolated from the TDLN (A), and intracellular staining of IFN- γ among CL4 cells isolated from the tumor (B). Histograms and dot plots are gated on Thy1.1⁺ CL4 cells collected from equal number of lymphocytes and total TIL. Numbers above the plots indicate the percentage of divided CL4 cells, and in the top left quadrants, the percentage of CL4 cells that are IFN- γ ⁺. The data are from one of three performed experiments. C, dot plots represent Ly6C expression on divided CL4 cells isolated from the TDLN of T3 and RencaHA tumor-bearing mice. Plots are gated on Thy1.1⁺ CL4 cells. The data are from one of two performed experiments, which used three animals per group. D, accumulation of dendritic cells within the tumors extracted from the T3- or RencaHA-bearing mice as described in A. Histogram shows percentage of CD11c⁺ cells among total cells collected. The data are from one of two performed experiments, which used three mice per group. Bars, SD. ***, $P < 0.001$.

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Previous studies in our laboratory have shown that RencaHA cells are able to directly prime CL4 CTL responses *in vitro*, leading to tumor cell lysis (20). To investigate whether the presence of PGE₂ during this interaction interferes with naïve CL4 CTL priming, RencaHA cells were cocultured for 72 hours with CFSE-labeled CL4 cells in the presence or absence of PGE₂. Following coculture, CL4 cells were isolated and analyzed for the expression of the activation markers CD69, CD28, and intracellular IFN- γ . RencaHA and K^dHA

peptide-pulsed splenocytes induced up-regulation of CD69, production of intracellular IFN- γ , and CD28 expression by CL4 cells, indicating the activation of these T cells (Fig. 5). As previously shown, CL4 cells cultured with HA-negative control RencaNT cells failed to up-regulate expression of any of these molecules, thus indicating that CL4 T-cell responses to RencaHA cells are K^dHA specific (20). Significantly, compared with CL4 cells cultured with RencaHA cells alone, proliferation, intracellular expression

Table 1. Use of COX-2 inhibitor, NS-398, up-regulates CL4 CTL responses to T3 tumor cells

	RencaHA (%)	T3 (%)	T3 +NS-398 (%)
CFSE ^{lo}	20	13	22
CFSE ^{lo} IFN-γ ⁺	5	1	7

NOTE: Thy1.2⁺ BALB/c mice were injected s.c. with 1×10^6 RencaHA or T3 tumor cells. On day 11, mice were given 3×10^6 CFSE-labeled, purified, naïve Thy1.1⁺ CL4 cells i.v. and on day 16 cells obtained from the TDLN were stained with antibodies against Thy1.1. Mice were injected i.p. with 10 mg/kg NS-398 before tumor injection and then thrice per week for the duration of the experiment, or left untreated. Numbers are indicative of the percentage of the divided cells (CFSE^{lo}) and of IFN-γ⁺ among divided cells (CFSE^{lo}IFN-γ⁺). The data are from one of two performed experiments, which used three animals per group.

of IFN-γ, and cell surface expression of CD28 was abrogated among CL4 cells cultured with RencaHA in the presence of PGE₂. However, the fact that these undivided CL4 cells up-regulated CD69 indicated that initial priming had occurred without inducing CL4 CTL responses (Fig. 5B-D).

Discussion

In this report, we show that PGE₂ abrogates both cross-presentation and direct presentation pathways involved in the priming of naïve tumor-specific CL4 CD8⁺ T-cell responses. The data show that whereas priming of naïve CL4 T cells by K^dHA

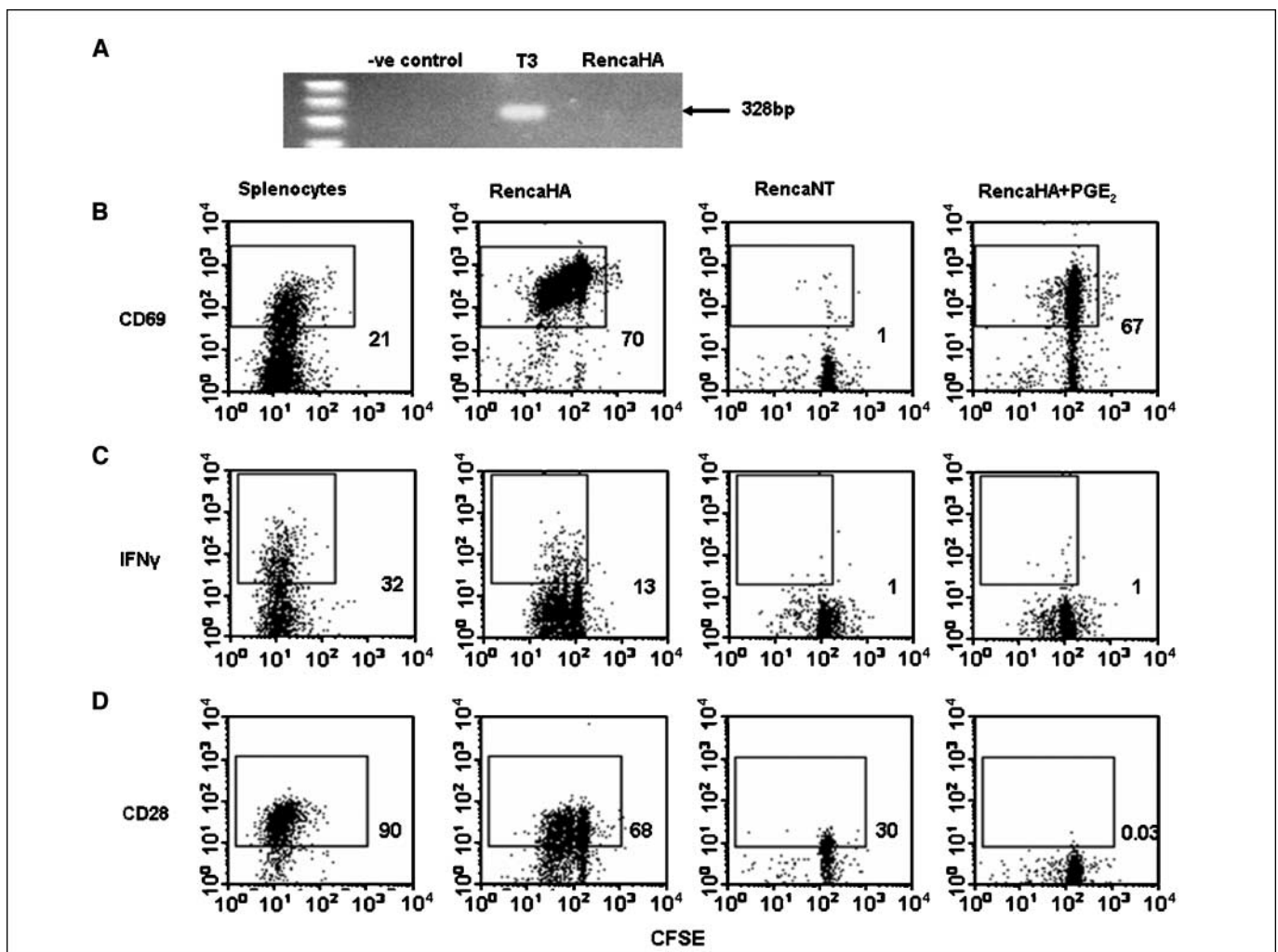


Figure 5. Exogenous PGE₂ abrogates direct activation of CL4 cells by tumor cells. TDLN of mice injected with RencaHA or T3 tumor cells followed by naïve CL4 cells, as described in Fig. 4, were excised on day 16 and analyzed for the presence of HA by RT-PCR (A). RencaHA, RencaNT, or peptide-pulsed splenocytes, in the presence or absence of PGE₂, were cocultured with naïve, CFSE-labeled Thy1.1⁺ CL4 cells for 72 h, and (B) CD69 expression, (C) IFN-γ production, and (D) CD28 expression by CL4 cells isolated from *in vitro* cultures were examined by flow cytometry. Dot plots are gated on Thy1.1⁺ CL4 cells and numbers indicate percentage divided CL4 cells expressing the receptor indicated in the figure. The data are from one of three performed experiments.

peptide-pulsed mDC resulted in their productive activation, coculturing with PGE₂-treated mDC resulted in reduced CL4 effector function, similar to that which occurs following priming by iDC. This finding is consistent with other studies that showed reduced APC function of dendritic cells, treated with PGE₂ (25). It is likely that the lack of productive activation of naïve CL4 T cells results from the immature phenotype of PGE₂-treated dendritic cells that were used as APC. Other *in vivo* studies from several laboratories, including our own, have shown that, whereas the interaction of antigen-loaded mDC with naïve CD8⁺ T cells activates effector CD8⁺ T-cell responses, cross-presentation of antigens by iDC to CD8⁺ T cells has been shown to induce tolerance (23, 33, 34). Furthermore, the abortive activation phenotype, displayed by CL4 cells within the TDLN of mice bearing T3 tumors, is consistent with the increased ability of PGE₂-treated iDC to migrate to TDLN (35). Although not tested in this study, it has been shown that increased migration of iDC to the TDLN is associated with an increase in the expression of CCR7 following PGE₂ treatment of iDC.

Our studies show that PGE₂ treatment of dendritic cells during their differentiation or maturation increases IL-10 production compared with mDC. This is in accordance with previous studies where PGE₂ was shown to induce IL-10 production by dendritic cells *in vitro* (6). Importantly, such an increase in IL-10 release by dendritic cells after exposure to PGE₂ may account for the lower numbers of TIDC in T3 tumors compared with RencaHA tumors (31). The importance of IL-10 in PGE₂-mediated inhibitory effects on dendritic cells was notable, as blocking of IL-10R during dendritic cell maturation reversed the effects of PGE₂ on APC function of dendritic cells. Consistent with other studies, fully matured dendritic cells were resistant to the effects of IL-10 (8, 25). We hypothesize that the type of CL4 CD8⁺ T-cell response generated by PGE₂-treated dendritic cells is determined at the site of dendritic cell maturation in the tumor microenvironment through increased production of IL-10. In addition, the data presented here may indicate a possible role of IL-10 in iDC-mediated abortive activation of CL4 cells and that blocking of IL-10R during iDC-CL4 interaction counters the ability of iDC to induce tolerance.

In vivo, COX-2 overexpression by T3 tumors rendered CL4 cells unable to proliferate, produce IFN- γ , or form TIL. Previous studies in our laboratory showed that *in vivo*, CL4 cell responses to RencaHA tumors rely on cross-presentation of HA antigen by BMDC (36). Data presented in this report show that overexpression of COX-2 enables T3 tumor cells to migrate to the

TDLN, indicating that T3 cells could also directly activate CL4 cells. However, the lack of CL4 cell activation in the TDLN of T3 tumor-bearing mice suggests that direct activation by T3 cells does not occur. Indeed, when RencaHA were cultured with CL4 cells *in vitro* in the presence of PGE₂ at a concentration comparable with that found within the tumor microenvironment of COX-2-overexpressing tumors (37), CL4 CTL responses were abolished. However, undivided CD8⁺ CL4 cells lost CD28 expression. The presence of CD8⁺ CD28⁻ T cells with regulatory functions has been observed in patients with different types of cancer (38, 39). Thus, in our system, a second stage of CD8⁺ T-cell tolerance could occur through direct interaction of T3 with CL4 cells.

Overall, our data suggest that COX-2/PGE₂ overexpression by tumors regulates both direct and indirect antigen presentation to naïve CD8⁺ T cells. We propose that PGE₂ within the tumor microenvironment both reduces the accumulation of TIDC, thus inhibiting antigen uptake, and maintains TIDC in an immature state that induces CD8⁺ T-cell tolerance upon migration to the TDLN. In addition, COX-2/PGE₂ overexpression by tumors allows migration of tumors to TDLN, also resulting in CD8⁺ T cell-tolerance induction. These findings provide us with an understanding of how PGE₂-overproducing tumor cells can regulate antitumor CD8⁺ T-cell responses. Critically, we and others have shown that dendritic cells that have already undergone maturation are refractory to the effects of PGE₂ and IL-10 (25). Therefore, immunotherapeutic strategies that use *ex vivo* generated mDC, rather than iDC (40), loaded with tumor antigen, would be preferable to prime protective antitumor CD8⁺ CTL response in patients with cancer. Furthermore, the use of PGE₂ inhibitors in patients with cancer would also be greatly desirable in promoting productive activation of antitumor CTL cell responses potentially generating fewer side effects than COX-2-specific inhibitors (41).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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