

# The immune modulation of Clara cell-10 in human peripheral monocytes and dendritic cells

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**Abstract.** Although Clara cell secretory protein (CC-10, CC-16 or uteroglobin, secretoglobin 1A1) has been ascribed anti-inflammatory, immunomodulatory and anti-cancer activity roles in lung diseases including lung cancer, its precise function remains unclear. The objective of the present study was to evaluate the role of CC-10 in the immunomodulation of human monocytes and dendritic cells (DCs). The human lung adenocarcinoma cell line A549, was used to examine PGE2 production after cyclooxygenase (COX) inhibition and adenovirus encoding human CC-10 cDNA (Ad-CC-10) transfection. Type I and II cytokines were measured from peripheral blood mononuclear cells (PBMCs) and DCs which were cultured with tumor supernatant (TSN) or Ad-CC-10 transfected TSN. When PBMCs were cultured with supernatant A549 (tumor supernatant, TSN), the levels of T-cell helper type 1 (Th1) and 2 (Th2) cytokines increased. However, CC-10 inhibited the induction of Th2 cytokines of PBMCs stimulated with TSN. In DCs, TSN inhibited Th1 type cytokines but induced Th2 type. In contrast, TSN treated with either CC-10 or NS398 (COX-2 inhibitor) stimulated Th1 type and inhibited Th2 type without any phenotypic changes. The supernatants generated in the presence of NS-398 or CC-10 prevented tumor-induced inhibition of allogeneic T-cell stimulation. While the level of interleukin (IL)-10 secretion from DC-Ad-CC-10 was decreased, the level of IL-12 secretion was increased by CC-10. Collectively our data suggest that a supernatant of NSCLC causes an imbalance in the immune response of PBMCs and DCs, which is reversed by CC-10.

This suggests that CC-10 is a candidate for the development of a new immunotherapy for lung cancer.

## Introduction

Tumor progression in normal immunocompetent individuals may reflect a failure of the immune system to recognize tumor antigens or may result from subversion of antitumor immune responses. Effective antitumor responses require both antigen-presenting cells and lymphocyte effectors. A defect in the host's immune system is one of the major mechanisms by which tumors evade immune surveillance and the suppressive milieu present within an established tumor inhibits the effective immune responses (1). New therapeutic strategies for cancer are clearly needed to manipulate the local tumor micro-environment and shift the balance back to a proinflammatory environment, which promotes dendritic cell (DC) activation, and enhances tumor immunity (2). There are several defects in the tumor environment. The tumor itself can secrete a variety of substances that either depress or inhibit the local immunity, such as interleukin-6 (IL-6) or transforming growth factor- $\beta$  (TGF- $\beta$ ), which can stop the proliferation of antigen specific T cells. Although cancer cells express tumor antigens, limited expression of MHC antigens, defective transporters associated with antigen processing, and lack of costimulatory molecules make them ineffective antigen-presenting cells. The active inhibition of a local inflammatory response by various T cell subsets adds to the immune inhibitory cytokine milieu that is characteristic of most human cancers (2,3). The cytokine profiles of cancer patients demonstrate an abnormal balance between the T-cell helper type 1 (Th1) and 2 (Th2) cytokines, favoring a Th2 response (1,4).

Cyclooxygenase-2 (COX-2) is constitutively overexpressed in a variety of epithelial malignancies including lung cancer (5-8). Although multiple genetic alterations are necessary for cancer invasion and metastasis, mounting evidence from numerous studies indicates that tumor COX-2 activity has a multifaceted role in conferring the malignant and metastatic phenotypes (8). COX-2 expression is rapidly induced as a secondary response to many factors, including growth factors, tumor promoters, and hormones (9).

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Overexpression of tumor COX-2 is associated with decreased host immunity (10), apoptosis resistance (11), increased angiogenesis (12), and enhanced invasion and metastasis (13). This can lead to the increased production of prostaglandins such as PGE<sub>2</sub>, which has multiple downstream effects. PGE<sub>2</sub> is known to transactivate the epidermal growth factor receptor, which in turn triggers mitogenic signaling in epithelial cells and induces cancer cell proliferation (14). PGE<sub>2</sub> also causes immunosuppression *in vitro* (15), and induces immunosuppression *in vivo*, thereby enhancing tumor growth in animal models (10,16).

Clara cell secretory protein (CC-10, CC-16 or uteroglobin, blastokinin, secretoglobin 1A1) is a steroid-inducible, evolutionarily conserved, low molecular mass (15.8 kDa) secretory protein that is synthesized by the mucosal epithelia from many organs including the thymus, pituitary gland, respiratory and gastrointestinal tracts, as well as the prostate and mammary glands (17). CC-10 was first discovered in rabbit uterine fluids as a critical element with a stimulatory effect on the growth of blastocysts during early pregnancy, and was named blastokinin (18). However, the physiological functions of CC-10 still remain elusive. CC-10 is the founding member of a growing superfamily of proteins called secretoglobin (Scgb) (19). Numerous studies have demonstrated that CC-10 is a multifunctional protein with anti-inflammatory (20) and immunomodulatory properties (21). It inhibits soluble phospholipase A<sub>2</sub> activity (22), binds and perhaps sequesters hydrophobic ligands such as progesterone, retinols, polychlorinated biphenyls, phospholipids and prostaglandins. In addition to its anti-inflammatory activities, CC-10 manifests anti-chemotactic (23), anti-allergic, anti-tumorigenic (24) and embryonic growth-stimulatory activities. Normal bronchial epithelia express the CC-10 gene at a high level. However, adenocarcinomas express CC-10 at a low level (25). In addition, the incidence of cancer in CC-10 knockout mice is extremely high. Induced expression of CC-10 in some cancer cells appears to cause the loss of their transformed phenotype and the suppression of metastasis (26). Overall, the loss of the CC-10 gene expression may be a common characteristic of cancer cells, and CC-10 may act as a tumor suppressor. It was reported that the induction of CC-10 gene expression has an inhibitory effect on COX-2 expression in lung cancer cells by suppressing NF- $\kappa$ B activity (27). CC-10 also has a function that suppresses the secretion of interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and IL-1 $\beta$ . However, it is unclear if CC-10 affects the host immune system in cancer. Based on its anti-inflammatory and anti-tumorigenic properties, CC-10 is a potential target for cancer immunotherapy.

In this study the role of CC-10 in the immune modulation for human monocytes and DCs was investigated with the hypothesis that CC-10 regulates the immune surveillance that tumors evade by secreting PGE<sub>2</sub>, which is a COX-2 product. We evaluated whether the tumor supernatant transduced by the adenovirus Ad-CC-10 has an effect on peripheral blood mononuclear cells (PBMC) as well as the functional and phenotypic changes in DCs transduced with Ad-CC-10.

## Materials and methods

**Cells line.** The human non-small cell lung carcinoma cell line, A549, was obtained from the American type Culture Collection

(Manassas, VA). Cells were maintained in a monolayer in RPMI-1640 (Irvine Scientific, Santa Anna, CA) containing 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), penicillin (60  $\mu$ g/ml) and streptomycin (100  $\mu$ g/ml) and 2 mM glutamine (JRH Biosciences, Lenexa, KS) at 37°C in an atmosphere containing 5% CO<sub>2</sub>. The cell line was mycoplasma-free and cells were used up to the tenth passage before thawing frozen stock cells from liquid N<sub>2</sub>. Cells were cultured in 75 ml plates to 80% confluence and were typically infected with purified Ad-CC-10 or Ad-null control vector for 48 h before harvesting and analysis.

**Reagents.** Cytokines used for the generation and stimulation of DCs included recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF), IL-4 and IFN- $\gamma$  (R&D Systems, Minneapolis, MN). Antibodies for phenotyping included fluorescently conjugated anti-CD14, anti-human leukocyte antigen (HLA)-DR, anti-CD83, and anti-CD86 (Becton Dickinson, San Jose, CA).

**Preparation of adenovirus expressing CC-10.** The adenoviral construct (Ad-CC-10) is an E1 deleted, replication-deficient adenoviral type 5 vector (Ad5) encoding human CC-10 cDNA. The recombinant Ad-CC-10 was constructed as described previously (26). Briefly, the cDNA of human CC-10 was cloned into the adenoviral shuttle vector, pACCMVpLpA. The pACCMVpLpA-CC-10 and the Invitrogen pJM17 adenoviral packaging plasmids were cotransfected into 293 cells (adenovirus E1a-transformed human embryonic kidney cells) by calcium phosphate/DNA coprecipitation. Homologous recombination between the two plasmids resulted in a recombinant replication-defective virus containing the human CC-10 gene. Viruses from 293-cell supernatants of cultures exhibiting a complete cytopathic effect were purified by CsCl purification. The control vector (Ad-null) did not contain the CC-10 cDNA insert. Clones of Ad-CC-10 were confirmed by CC-10-specific ELISA. A large-scale stock of the adenovirus was prepared using the standard CsCl purification, dialysis, and storage as a glycerol (10% v/v) stock at -80°C. The titer of each viral stock was routinely 10<sup>11</sup>-10<sup>13</sup> plaque forming units by plaque assay.

**Preparation of conditioned medium.** Tumor supernatants (TSN) were collected from A549 cells (1x10<sup>6</sup> cells/ml) following a 48-h culture in culture medium. Supernatants were also collected from cells treated with DMSO or a COX inhibitor, NS-398 (10  $\mu$ M) (Cayman Biochemical Co., CA), and from Ad-null or Ad-CC-10 transfected cells (20 MOI). These conditioned culture media were stored at -70°C until needed.

**PGE<sub>2</sub> enzyme immunoassay.** PGE<sub>2</sub> concentrations were determined using a kit from Cayman Chemical Co. (Ann Arbor, MI) according to the manufacturer's instructions. The enzyme immunoassay plates were read by a Molecular Dynamics Microplate Reader (Sunnyvale, CA). TSN treated with TNF- $\alpha$  (5 ng/ml) (Invitrogen, Carlsbad, CA) for 1 h was used as the positive control. NS-398 was used as the standard inhibitor.

**Isolation and culture of peripheral blood monocytes.** The use of a leukocyte-enriched buffy coat from patients and healthy donors was approved by the Seoul National University Hospital

Review Board, and informed consent was obtained from all donors. PBMCs were obtained from a leukocyte-enriched buffy coat by centrifugation with Ficoll-Hystopaque™ (Sigma Diagnostics, Inc., St. Louis, MO). Cells were resuspended at  $2 \times 10^6$  cells/ml in RPMI-1640 supplemented with 20 mM HEPES buffer (Gibco, Carlsbad, CA), penicillin-streptomycin, and 2 mM glutamine. Cells were allowed to adhere to the tissue culture flasks for 2 h at 37°C in an atmosphere containing 5% CO<sub>2</sub>. After incubation, the non-adherent cells were removed, and remaining adherent cells were washed twice in PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Gibco). PBMCs were allowed to adhere for 4 h at 37°C, 5% CO<sub>2</sub> and the non-adherent cells were washed away with PBS. Adherent cells were cultured in 50% complete medium (CM) for 2 more days.

**Cytokine assay.** Commercial kits were used to measure human IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-10 and TGF- $\beta$  levels (sensitivities, 10, 16, 32, 10 and 16 pg/ml, respectively; R&D Systems) in PBMCs, and human IL-12, IL-10, TGF- $\beta$ , and IL-6 levels in DCs (sensitivities, 10, 10, 16, and 10 pg/ml, respectively; R&D Systems).

**Generation of human monocyte-derived DCs.** DCs were derived from PBMCs. PBMCs from a leukocyte-enriched buffy coat from healthy donors or patients were cultured for 6 days in complete RPMI medium containing 50% complete RPMI medium supplemented with 10% human serum AB (Cambrex, Walkersville, MD), recombinant human GM-CSF (75 ng/ml) and recombinant human IL-4 (75 ng/ml) to induce the monocyte-derived DC at 37°C in an atmosphere containing 5% CO<sub>2</sub>. rhGM-CSF and rhIL-4 were added every 3 days during culture.

**Immunophenotypic analysis of DCs by flow cytometry.** The DC phenotype was analyzed on day 8 of culture. Briefly, cultured cells were stained directly with the following monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), FITC-CD14, HLA-DR, and CD83, PE-CD86. Cells were also stained with the corresponding FITC or PE-conjugated, isotype-matched control antibody. Washed DCs were labeled with the fluorochrome-conjugated monoclonal antibody for 30 min on ice. Cells were washed twice in a FACS buffer (PBS, 2% FBS) and analyzed using a FACS flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software in the Seoul National University Hospital Clinical Research Institute, Seoul, Flow Cytometry Core Facility. Data analysis was performed by CellQuest software.

**Mixed lymphocyte reaction.** A total of  $5 \times 10^4$  responder PBMCs per well, which were isolated from buffy coats as described above, were incubated with titrated amounts of the allogeneic stimulator DCs. Responders and stimulators were cultured in 96-well plates containing complete medium for 5 days at 37°C and 5% CO<sub>2</sub>. The level of cell proliferation was determined using a bromodeoxyuridine (BrdU) incorporation ELISA kit (Roche Diagnostics GmbH, Germany).

**Transduction of DCs with gene-modified adenovirus.** To optimize the multiplicity of infection for CC-10 production, *in vitro* propagated monocyte-derived DCs were transduced

with human Ad-null or Ad-CC-10 on day 6 of culture using the standard method or with butyrate (200 mM) at MOI of 10:1, 50:1 and 100:1. Briefly,  $1.0 \times 10^6$  DCs were transduced at 50 or 100 MOI by an adenovirus in serum-free RPMI for 2 h with gentle shaking. DCs, DC-Ad-null and DC-Ad-CC-10 were washed twice and incubated with CM at 37°C for an additional 2 days. Efficiency of transduction of DCs by adenovirus type 5 vector was assessed with an Ad5LacZ vector. The transduced DCs produced 7.9-10.7 ng/ml/ $10^6$  cells/24 h of CC-10.

**CC-10 ELISA.** CC-10 protein secretion was determined from DCs, DC-Ad-null and DC-Ad-CC-10 culture supernatants using CC-10-specific ELISA kit (United States Bio, Swampscott, MA) according to the manufacturer's instructions. Briefly, CC-10 standard protein or DC culture supernatants (derived from  $1.0 \times 10^6$  cells/1.0 ml) were added to each well of an ELISA plate precoated with an anti-CC-10 Ab. After 2 h incubation at room temperature, the plate was washed four times and an HRP-conjugated anti-CC-10 secondary Ab was added to each well. After 2 h incubation at room temperature, the plate was washed four times to remove all unbound reagents and 3,3',5,5'-tetramethyl-benzidine substrate buffer was added to each well. Reactions were stopped by adding 1 M sulfuric acid and the OD was read at 450 nm by a Molecular Dynamics Microplate Reader (Sunnyvale, CA). BAL fluid was used as the positive control. The sensitivity of the CC-10 ELISA assay was 200 pg/ml.

**Chemotaxis assay.** Transwell cell-migration plates (Corning Costar, Cambridge, NY) were used for the chemotaxis assay to evaluate the biological function of the supernatant derived from DCs, DC-Ad-null and DC-Ad-CC-10. The 12-well transwell plates were equipped with inserts whose bottoms were sealed with polycarbonate membranes with 3- $\mu$ m pores. Wells of transwell plates were filled with 0.6 ml of bottom solution (serum-free medium). Isolated PBL cells ( $2 \times 10^5$ ) were suspended in 100  $\mu$ l serum-free medium and pipetted into the insert, and the insert was transferred into the well. Cell migration was stimulated by the supernatant from  $1 \times 10^6$  DCs, DC-Ad-null and DC-Ad-CC-10, present in the bottom solution. AB medium (10%) was used as the negative control. The positive control medium contained the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP,  $10^{-8}$  M, Sigma Diagnostics, Inc., St. Louis, MO). Transwell plates were incubated in a humidified CO<sub>2</sub> incubator at 37°C during the migration (2 h). After top solution was removed, the non-migrating cells were aspirated gently from the upper chamber, which was further wiped with a cotton swab. The cells on the bottom surface of the chamber were collected and washed twice with PBS and fixed in 70% methanol and stained in 1% Coomassie Brilliant Blue. Cells that migrated and adhered to the lower surface were counted.

**Cytokine assay of transduced-DC.** Human IL-12 production was induced by priming  $1.0 \times 10^5$  DCs, DC-Ad-null and DC-Ad-CC-10 with 50 ng/ml IFN- $\gamma$  for 2 h, and then stimulating them with 1  $\mu$ g/ml LPS (Sigma) for 48 h. Human IL-10 production was induced by stimulating the  $1.0 \times 10^5$  DCs, DC-Ad-null and DC-Ad-CC-10 with 1  $\mu$ g/ml LPS for



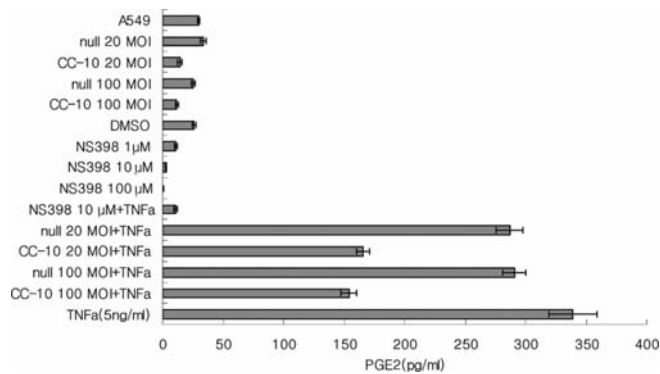


Figure 1. CC-10 inhibits PGE2 synthesis and release. The secretory PGE2 concentrations were determined using an enzyme immunoassay system. The level of PGE2 in the medium from lung cancer cells revealed a significant amount of synthesis and release of PGE2 in these cells. PGE2 in TSN transduced with CC-10 was reduced. TSN treated with TNF- $\alpha$  (5 ng/ml) for 1 h was used as the positive control. NS-398 was used as the standard inhibitor.

48 h. IL-12 and IL-10 concentrations in the supernatant of either the unstimulated or stimulated DCs were measured by specific ELISA according to the manufacturer's instructions. The sensitivity of ELISA for both IL-12 and IL-10 was 10 pg/ml.

**Statistical analysis.** A paired t-test was used to compare the differences in the various CM exposed PBMC. An unpaired two-tailed Student's t-test was used to compare the differences in the inhibitor treated DCs. The level of statistical significance was set at  $P < 0.05$ .

## Results

**CC-10 inhibits PGE2 production from NSCLC.** In order to determine if CC-10 regulated the production of PGE2 from NSCLC cells, secretory PGE2 concentrations were determined using a kit from Cayman Chemical Co. according to the manufacturer's instructions. As shown in Fig. 1, PGE2 in the TSN from A549 cells transduced with Ad-CC-10 was markedly decreased compared to those in the naïve A549 TSN or in the Ad-null transduced A549 TSN. Similar findings were observed after TNF- $\alpha$  stimulation increasing the level of PGE2 production. However, NS-398, a COX-2 inhibitor, reduced the level of PGE2 production in a dose-dependent manner. Western blot analysis confirmed Ad-CC-10 transduced A549 cells and induced CC-10 (data not shown).

**CC-10 inhibits the induction of Th2 cytokines of PBMC stimulated by TSN.** To determine if naïve TSN or TSN transduced with Ad-CC-10 has an effect on PBMCs, secretory cytokines from PBMCs were determined. The measured cytokine levels were standardized by the basal level of the negative control, which was PBMCs cultured in complete culture medium, expressed by the fold increase (Fig. 2). Both Th1 type cytokines such as IL-2 (Fig. 2A), IFN- $\gamma$  (Fig. 2B) and TNF- $\alpha$  (Fig. 2C), and Th2 type cytokines such as IL-10 (Fig. 2D) and TGF- $\beta$  (Fig. 2E), were induced when PBMCs were co-cultured with the supernatant from NSCLC cells.

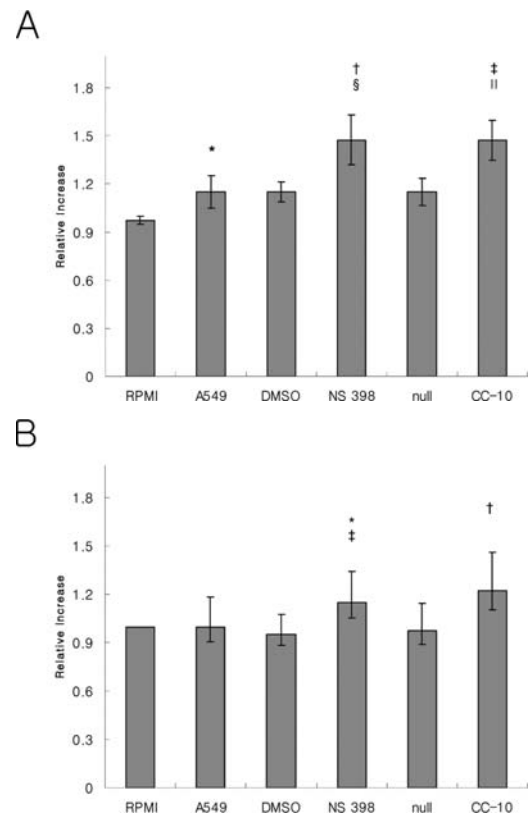


Figure 2. CC-10 inhibits the induction of Th2 cytokines of PBMCs stimulated by TSN. The secretory cytokines from PBMCs cultured with various TSN were determined by ELISA. Both Th1 type cytokines such as IL-2 (A), IFN- $\gamma$  (B) and TNF- $\alpha$  (C), and Th2 type cytokines such as IL-10 (D) and TGF- $\beta$  (E), were induced when PBMCs were co-cultured with the supernatant from NSCLC cells. However, the CC-10 and NS-398, COX-2 inhibitor, did not induce Th2 cytokines from PBMCs stimulated by TSN, showing that the level of Th1 type cytokines was increased by CC-10, but Th2 type cytokines was decreased. RPMI, complete medium; A549, tumor supernatant; DMSO, A549 treated with DMSO; NS398, A549 treated with NS398 10  $\mu$ M; null, A549 treated with Ad-Null 20 MOI; CC-10, A549 treated with Ad-CC-10 20 MOI.  $P < 0.05$ ; \*RPMI vs. A549;  $^{\dagger}$ DMSO vs. NS398;  $^{\ddagger}$ null vs. CC-10;  $^{\S}$ A549 vs. NS398;  $^{\parallel}$ A549 vs. CC-10.

However, the CC-10 and NS-398, COX-2 inhibitor, did not induce the Th2 cytokine from PBMCs stimulated by TSN, showing that the level of Th1 type cytokines was increased by CC-10, but Th2 type cytokines were decreased.

**DC phenotypes were not altered by culturing in conditioned medium.** The expression of DC surface markers was not altered significantly when DCs were co-cultured with TSN or NS-398 or CC-10-treated TSN. The immature DC phenotype was preserved in CM without altering CD80, CD83 and HLA-DR expression (Fig. 3). The expression of CD14, which is marker of monocytes, was used as the negative control.

**CC-10 inhibits the induction of Th2 cytokines from DC stimulated by TSN.** To determine if naïve TSN or TSN transduced with Ad-CC-10 had an effect on DCs, secretory cytokines from DCs were measured (Fig. 4). In contrast to the PBMC results, the level of the Th1 type cytokines such as IL-12 (Fig. 4A) decreased when the DC were cultured with a supernatant of non-small lung cancer cells. However, the

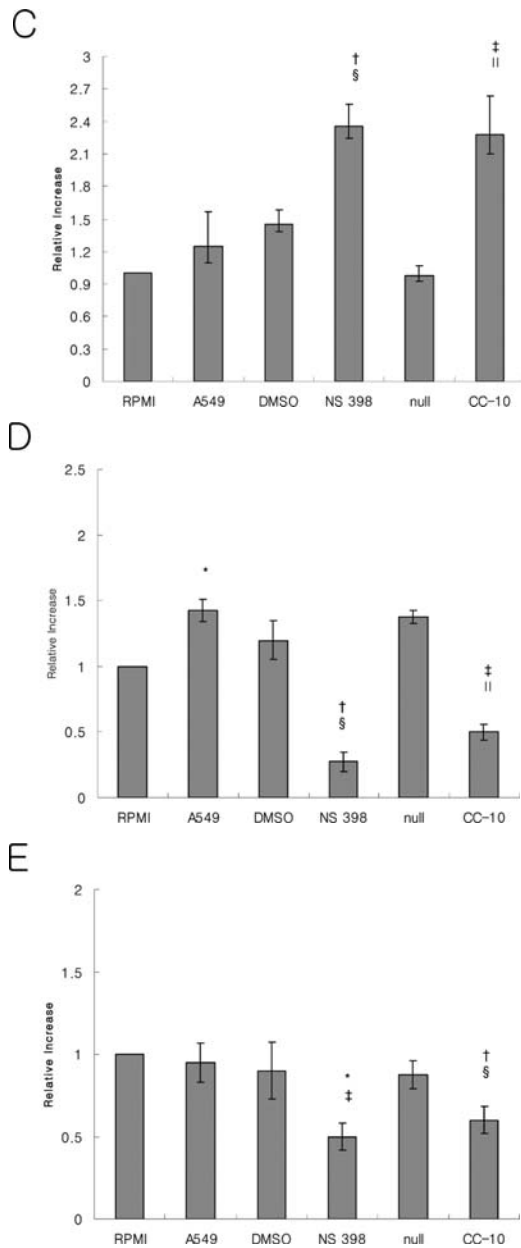


Figure 2 continued.

level of Th2 type cytokines such as IL-10 (Fig. 4B), TGF- $\beta$  (Fig. 4C) and IL-6 (Fig. 4D) were increased. CC-10 and NS-398 did not induce Th2 cytokine from DCs stimulated by TSN, showing that the level of Th1 type cytokines was increased by CC-10, but that of Th2 type was decreased.

**CC-10 leads T cell stimulatory capacity of DC differentiation in conditioned medium.** Having established the inhibitory effect of the tumor-derived soluble factors on the development of DCs, the effects of tumor-derived soluble factors on DCs generated in their presence to stimulate T cells in an allogeneic mixed lymphocyte reaction (MLR) mixtures were evaluated. A significant inhibitory effect was observed in DCs cultured in TSN. A slight but consistent stimulatory effect was observed in MLR when TSN-treated positive control was added during Mo-DC generation. In contrast, the supernatants generated in the presence of NS-398 or CC-10

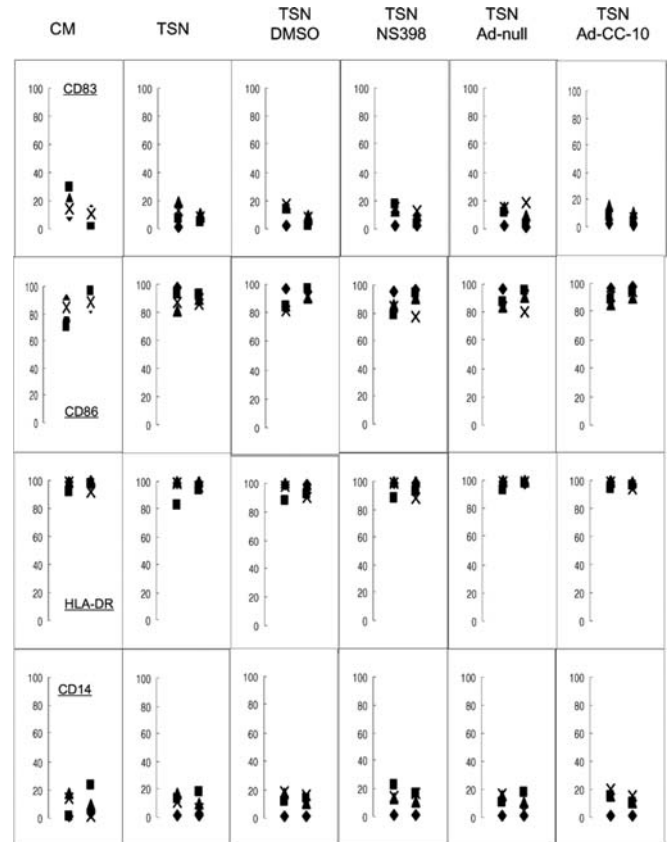


Figure 3. DC phenotypes are not altered by culturing in the conditioned medium. DCs in the complete medium and various conditioned media were characterized by flow cytometry after 8 days of culture. The expression of DC surface markers was not significantly altered in the culture with TSN or NS-398 or CC-10 treated TSN. Immature DC phenotypes were preserved in CM without altering the CD80, CD83 and HLA-DR levels compared with the control (left panels) and patients (right panels).

abrogated the tumor-induced inhibition of allogeneic T-cell stimulation (Fig. 5).

**Ad-CC-10 gene-modified human DCs produce biologically active levels of CC-10.** On day 6 of culture, the immature human monocyte-derived DCs ( $10^6$  cells/ml) were transduced with Ad-CC-10 for 48 h at various MOIs (20:1-500:1). Transduction was accomplished using the standard method (2 h incubation at  $37^\circ\text{C}$ ), as described previously (26). The production of the CC-10 protein was assessed by ELISA specific for human CC-10, and a higher concentration was demonstrated with increasing MOI, showing a 10-fold increase in CC-10 protein secretion with 50 MOI and a 15-fold increase with 100 MOI compared with the negative control (Fig. 6). Cell viability decreased significantly at MOI  $>300:1$  (data not shown). There were no significant differences noted in the level of CC-10 protein production compared with the transduction observed using butyrate or the LPS-matured DCs (data not shown). For subsequent studies, the DC phenotype and function were evaluated 48 h after Ad-CC-10 transduction in immature DCs with 50 MOI.

**DC phenotypes are maintained after transduction with Ad-CC-10.** The phenotypes of DC, DC-Ad-null and DC-Ad-CC-10 were evaluated by flow cytometry 48 h after transduction.

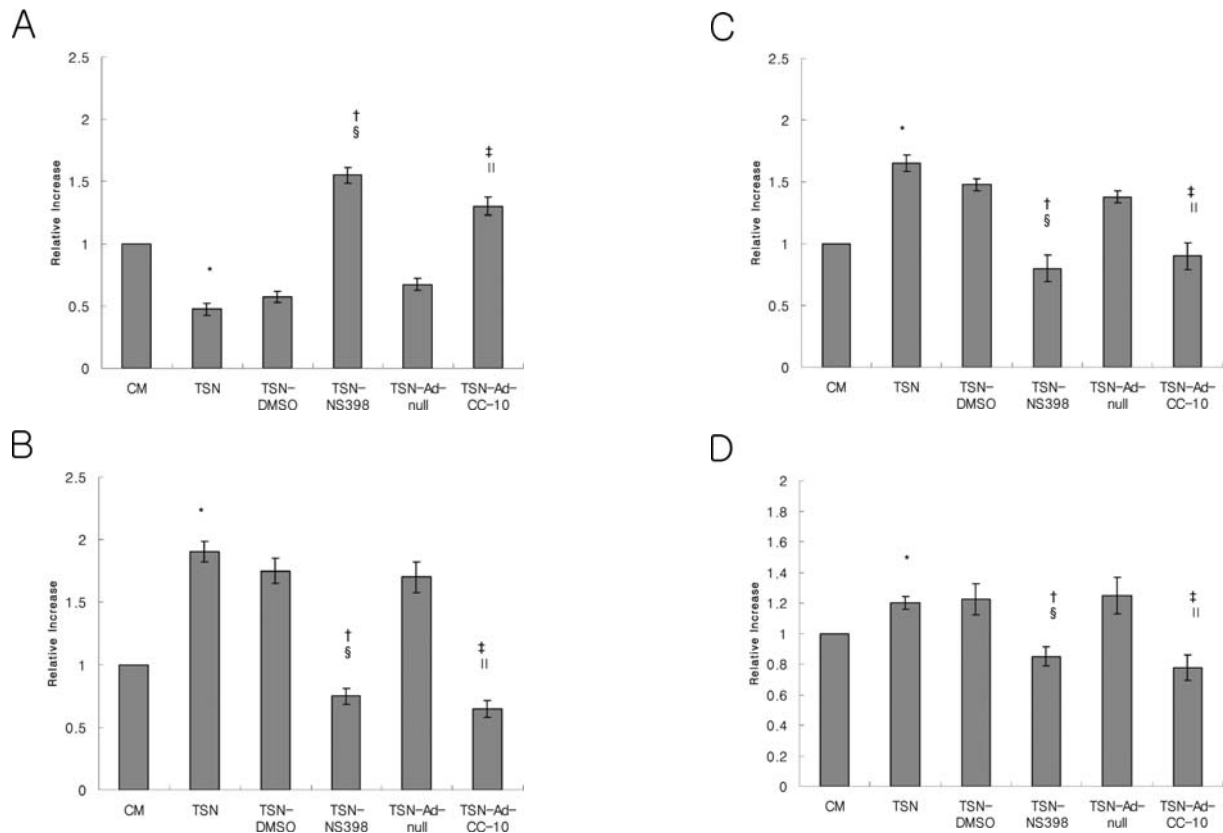


Figure 4. CC-10 inhibits the induction of Th2 cytokines from DCs stimulated by TSN. Cytokines of DCs cultured with various TSN were analyzed by ELISA. In contrast to the PBMC results, the level of Th1 type cytokines such as IL-12 (A) was reduced when DCs were cultured with the supernatant of non-small lung cancer cells but the level of Th2 type cytokines such as IL-10 (B), TGF-β (C) and IL-6 (D) increased. CC-10 and NS-398 induced a normal immune response in DCs. Although the Th1 type cytokines were up-regulated by CC-10, the Th2 type cytokines were down-regulated. CM, complete medium; TSN, tumor supernatant; TSN-DMSO, A549 treated with DMSO; TSN-NS398, A549 treated with NS398 10 μM; TSN-Ad-null, A549 treated with Ad-Null 20 MOI; TSN-Ad-CC-10, A549 treated with Ad-CC-10 20 MOI.  $P < 0.05$ ; \*RPMI vs. A549; †DMSO vs. NS398; ‡null vs. CC-10; §A549 vs. NS398; ||A549 vs. CC-10.

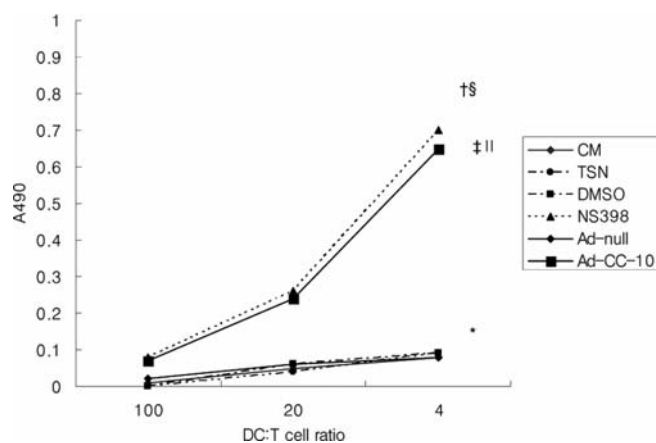


Figure 5. T cell stimulatory capacity of DCs is differentiated in conditioned medium. The T cell stimulatory capacity of DCs was analyzed using a mixed lymphocyte reaction (MLR). A significant inhibitory effect was observed in DCs cultured in TSN. A slight but consistent stimulatory effect was observed in MLR when TSN-treated positive control was added during Mo-DC generation. In contrast, supernatants generated in the presence of NS-398 or CC-10 led to an abrogation of the tumor-induced inhibition of allogeneic T-cell stimulation. RPMI, complete medium; A549, tumor supernatant; DMSO, A549 treated with DMSO; NS398, A549 treated with NS398 10 μM; Ad-null, A549 treated with Ad-Null 20 MOI; Ad-CC-10, A549 treated with Ad-CC-10 20 MOI. All  $P < 0.05$ ; \*RPMI vs. A549; †DMSO vs. NS398; ‡null vs. CC-10; §A549 vs. NS398; ||A549 vs. CC-10.

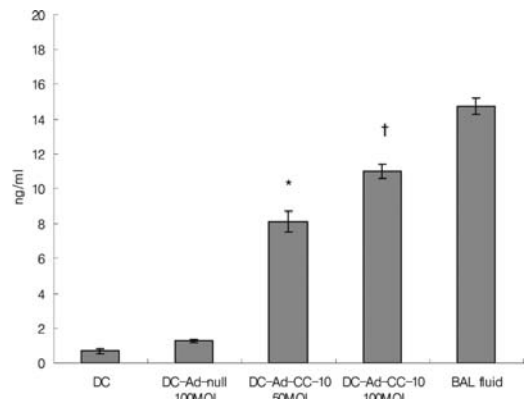


Figure 6. Ad-CC-10 gene-modified human DCs produce biologically active levels of CC-10. CC-10 protein concentration from DCs, DC-Ad-null and DC-Ad-CC-10 supernatants were determined by CC-10-specific ELISA. BAL fluid was used as the positive control. The protein production increased with increasing MOIs, and reached a plateau at MOI >100:1.  $P < 0.05$ ; \*DC-Ad-null 100 MOI vs. DC-Ad-CC-10 50 MOI; †DC-Ad-CC-10 50 MOI vs. DC-Ad-CC-10 100 MOI.

The surface marker expression of DCs was not altered significantly by transduction with either Ad-CC-10 or Ad-null (Fig. 7). The immature DC phenotype was preserved in the DC-Ad-null and DC-Ad-CC-10 without up-regulation of

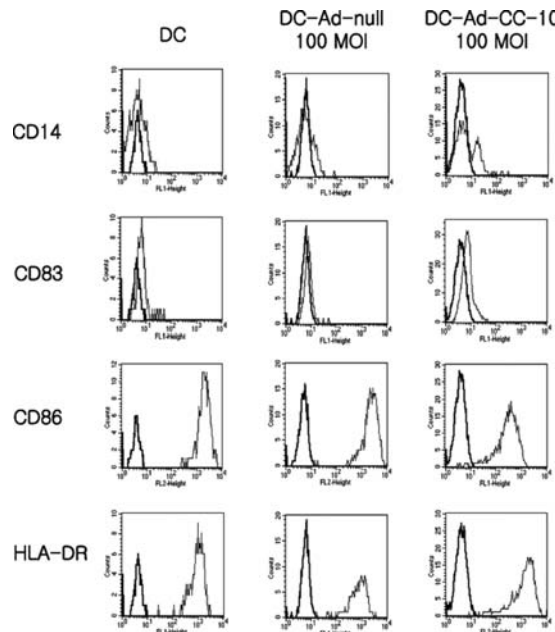


Figure 7. DC phenotypes were maintained after transduction with Ad-CC-10. DC, DC-Ad-null and DC-Ad-CC-10 were characterized by flow cytometry 48 h after transduction. The expression of the DC surface markers was not significantly altered by transduction with Ad-CC-10 or Ad-null. The immature DC phenotype was preserved in DC-Ad-null and DC-Ad-CC-10 without any up-regulation of CD83, CD86, or HLA-DR. There was no significant difference in CD14 expression between the DC, DC-Ad-null and DC-Ad-CC-10.

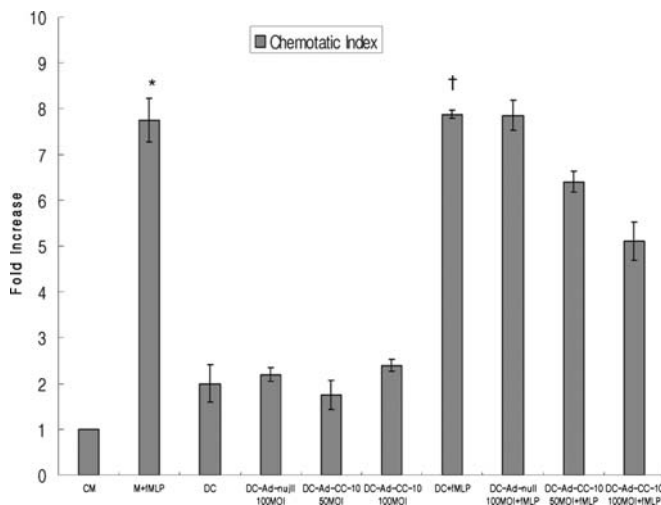


Figure 8. DC-Ad-CC-10 has no effect on chemotaxis of PBL. Migration of peripheral blood lymphocytes (PBL) to the DC-Ad-UG was assessed using a standard transwell chemotaxis assay. Supernatants from DCs, DC-Ad-null and DC-Ad-CC-10 were used. PBL gave a good response to the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP,  $10^{-8}$  M). However, DCs, DC-Ad-null and DC-Ad-CC-10 were not affected by the chemotaxis of PBL.  $P < 0.05$ ; \*Medium vs. Medium + fMLP; †DC vs. DC + fMLP.

CD83, CD86, or HLA-DR. There was a similar level of CD14 expression in DCs, DC-Ad-null and DC-Ad-CC-10.

*DC-Ad-CC-10 has no effect on chemotaxis of PBL.* A transwell migration assay where supernatants from DC, DC-Ad-null

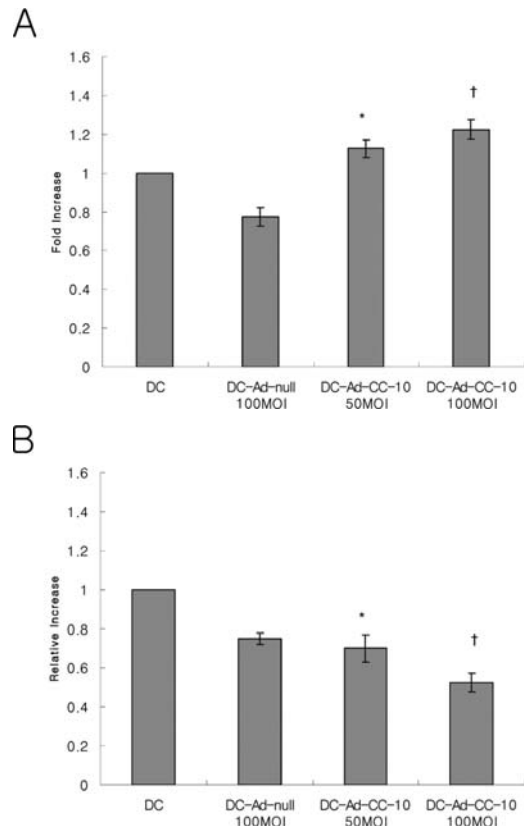


Figure 9. Ad-CC-10 transduction enhances Th1 type cytokine production. ELISA was used to analyze the affect of Ad-CC-10 and Ad-null transduction on DC production of IL-12 and IL-10 after stimulation with IFN- $\gamma$  and LPS or LPS alone, respectively. An unpaired two-tailed Student's t-test was used to compare the differences using SPSS. DC-Ad-CC-10 increased the capacity of DCs to secrete IL-12 with a concomitant decrease in IL-10 production.  $P < 0.05$ ; \*DC-Ad-Null 100 MOI vs. DC-Ad-CC-10 50 MOI; †DC-Ad-CC-10 50 MOI vs. DC-Ad-CC-10 100 MOI.

and DC-Ad-CC-10 or fLMP, added as a chemotactic factor, were used to determine if DC-Ad-CC-10 alters migratory behavior in peripheral blood lymphocytes (PBL). Freshly isolated PBLs ( $2 \times 10^5$ ) were allowed to migrate through  $3\text{-}\mu\text{m}$  pore inserts of the transwell plate. The total cell migration was determined by counting the number of cells. PBL gave a good response to the chemoattractant, fMLP. However, the chemotaxis of PBL was not affected by DCs, DC-Ad-null and DC-Ad-CC-10 (Fig. 8).

*Ad-CC-10 transduction enhances Th1 type cytokine production.* Previous studies have suggested that adenoviral vector transduction of DC stimulates the enhanced effector cell activity, partly through the increase in IL-12 production (Fig. 9). Production of IL-12 (Fig. 9A) and IL-10 (Fig. 9B) by DC-Ad-CC-10 after stimulation with IFN- $\gamma$  and LPS or LPS alone were evaluated. DC transduced by Ad-CC-10 enhanced the IL-12 secretion with a concomitant decrease in IL-10 production.

## Discussion

Complex interactions between the tumor and the host immune system contribute to the development of immunosuppressive networks within the tumor milieu. There is evidence suggesting



that tumor-specific antigens are present in cancer cells that can function as potential targets for the immune system. Tumor progression in normal immunocompetent individuals may reflect a failure of the immune system to recognize tumor antigens or may result from subversion of antitumor immune responses. Cancer patients do not mount an effective immune response, indicating that the immune cells have become tolerant to the tumor-specific antigens. Tumors also exhibit multiple immunosuppressive strategies, such as the secretion of immunosuppressive cytokines, as well as the production of high levels of COX-2 and PGE2 (1).

CC-10 appeared to have a high homology with rabbit uteroglobin, which has immunosuppressive, anti-inflammatory, anti-proteinase, and anti-phospholipase A2 properties (20-24). Therefore, CC-10 might play an important role in anti-inflammatory response which is a critical factor for immune modulation in cancer prevention and treatment and CC-10 may be used for a new marker indicating the severity of various inflammatory lung diseases (34). Chen *et al* recently reported that former smokers had higher plasma and BAL levels of CC-10 than current smokers, indicating that at least some of the damage associated with tobacco smoke may be repaired by long-term smoking cessation (36). The goal of the present study was to determine the anti-inflammatory effect of CC-10, protein secreted by bronchiolar Clara cells that is abundantly present in BAL and measurable in blood, in the immunomodulation of lung cancer. In this study, we show that CC-10 plays an important role in anti-inflammatory response in PBMCs or DCs. As described before, CC-10 suppressed COX-2 expression via inhibition of NF- $\kappa$ B activity in lung cancer cells (27), CC-10 caused the reduction of PGE2 production from NSCLC and reduced the immunosuppressive cytokine secretion from PBMC or DC which were stimulated with TSN.

PGE2 is an immune suppressor that targets both the cytotoxic and helper T-cell functions. PGE2 is believed to suppress cell-mediated immune responses while enhancing the humoral immune responses (28,29). It was reported that the induction of CC-10 gene expression has an inhibitory effect on the COX-2 expression in lung cancer cells via the suppression of NF- $\kappa$ B activity (27). In this study, an analysis of PGE2 in the medium from the A549 cells revealed a significant amount of PGE2 synthesis and release. However, CC-10 inhibited the synthesis and release of PGE2. NS-398, COX-2 inhibitor, as the positive control, reduced the level of PGE2 production in a dose-dependent manner.

PGE2 suppresses the production of chemokines and cytokines in humans, including IFN- $\gamma$ , TNF- $\alpha$ , IL-12, and the IL-1-mediated expression of chemokines. PGE2 up-regulates the expression of the immunosuppressive cytokines, such as IL-10 and TGF- $\beta$  (30,31). This immunosuppressive effect of PGE2 was demonstrated by the inhibition of normal T-cell proliferation to various tumor lysates with a PGE2 high concentration (1). Sharma *et al* reported that PGE2 upset the balance of Th1/Th2 cytokines (31). Therefore, this study investigated TSN or TSN transduced with the CC-10 affected PBMC. CC-10 induced normal balance between Th1 and Th2 cytokines in PBMCs. The levels of both the Th1 and Th2 type cytokines increased when the PBMC were cultured with a supernatant of non small lung cancer cells. CC-10 and

NS-398 induced the normal immune response of PBMC. Although the Th1 type cytokines were up-regulated by CC-10, the Th2 type cytokines were down-regulated.

The methods for generating anticancer immunity must focus on the initiation and maintenance of an inflammatory environment complete with APCs with sufficient potency to stimulate naive T cells, i.e., DCs. DCs are unique in their expression of important costimulatory molecules and their ability to process and present self-antigens (2). It was reported that DCs cultured in TSN failed to generate an antitumor immune response and had immunosuppressive effects that correlated with enhanced tumor growth. However, the genetic or pharmacological inhibition of tumor-COX-2 expression restored the function of DCs and the effective antitumor immune responses (31). In our study, we showed that the level of Th1 cytokines were reduced when PBMCs cultured with TSN but the levels of Th2 cytokines increased. CC-10 and NS-398 induced a normal immune response in DCs. Although Th1 type cytokines were up-regulated by CC-10, Th2 type cytokines were down-regulated. In addition, the expression of the DC surface markers was not significantly altered in cells cultured with TSN, or NS-398 or CC-10-treated TSN. Overall, the tumor-derived soluble factors have an inhibitory effect on DC development. Therefore, their effect on the ability of DCs generated in their presence to stimulate T cells in an allogeneic MLR was next evaluated. A considerable inhibitory effect was observed in DCs cultured in TSN. In contrast, the supernatants generated in the presence of either NS-398 or CC-10 repressed the tumor-induced inhibition of allogeneic T-cell stimulation.

The ability of DCs to capture, process and present antigens with the subsequent activation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes makes them ideal adjuvants in immunotherapy approaches to cancer. In animal models, an intratumoral injection of Ad-transduced DC expressing the cytokine genes was reported to generate enhanced anti-tumor effects compared with DCs or DCs transduced with the control vector (31-33). DCs were characterized after being transduced with an adenoviral vector expressing the CC-10 gene. At the MOIs evaluated, DCs maintained their immature phenotype without significantly up-regulating the DC marker. It was recently reported that high concentrations of PGE2 caused a decrease in IL-12 production via an increase in IL-10 production with a concomitant decrease in the DC function (30). This correlates well with our data, which clearly demonstrated an increase in the PGE2 levels within the tumor milieu as well as an increase in the intracellular IL-10 levels and a decrease in the IL-12 levels. However, Ad-CC-10 transduction decreased the level of the Th-2 type cytokines with a concomitant increase in the production of Th1 type cytokine in DC. This suggests that the Th1 cytokine-induced Ad-CC-10 promotes the development of the cell-mediated anti-tumor responses.

PGE2 was overexpressed in the supernatant of the NSCLC and the effect of CC-10 was observed. It is believed that mediators of immune suppression, such as COX-2 and PGE2, can be inhibited if the cytotoxic T-lymphocyte effector functions can be costimulated with the appropriate immune-based therapy to overcome the immune-system resistant effects of the tumor (1). This study, along with other studies reported in the literature, provides us with an immunological rationale



for inhibiting COX-2 to reduce the PGE2 levels as well as the level of immunosuppression and tumor cell growth. CC-10 affected the suppression of the abnormal immune system in NSCLC by inhibiting PGE2.

In summary, a supernatant of NSCLC caused an imbalance in the immune response of PBMCs and DCs, which was reversed by CC-10. Moreover, CC-10 changed the pattern of cytokine secretion from DC without adversely affecting the DC phenotype. The results from this study suggest that CC-10 has an immune modulation effect on immune effector cells and APCs, causing augmentation of the antitumor responses. The potent immune modulation effects of CC-10 provide a strong rationale for additional evaluation of CC-10 in the regulation of tumor immunity and development of a new immune therapy for lung cancer.

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