

Intrapulmonary Administration of CCL21 Gene-Modified Dendritic Cells Reduces Tumor Burden in Spontaneous Murine Bronchoalveolar Cell Carcinoma

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Abstract

The antitumor efficiency of dendritic cells transduced with an adenovirus vector expressing secondary lymphoid chemokine (CCL21) was evaluated in a murine model of spontaneous bronchoalveolar cell carcinoma. The transgenic mice (CC-10 TAG) express the SV40 large T antigen (TAg) under the Clara cell promoter, develop bilateral, multifocal, and pulmonary adenocarcinomas, and die at 4 months as a result of progressive pulmonary tumor burden. A single intratracheal administration of CCL21 gene-modified dendritic cells (DC-AdCCL21) led to a marked reduction in tumor burden with extensive mononuclear cell infiltration of the tumors. The reduction in tumor burden was accompanied by the enhanced elaboration of type 1 cytokines [IFN- γ , interleukin (IL)-12, and granulocyte macrophage colony-stimulating factor] and antiangiogenic chemokines (CXCL9 and CXCL10) but a concomitant decrease in the immunosuppressive molecules (IL-10, transforming growth factor- β , prostaglandin E₂) in the tumor microenvironment. The DC-AdCCL21 therapy group revealed a significantly greater frequency of tumor-specific T cells releasing IFN- γ compared with the controls. Continuous therapy with weekly intranasal delivery of DC-AdCCL21 significantly prolonged median survival by >7 weeks in CC-10 TAG mice. Both innate natural killer and specific T-cell antitumor responses significantly increased following DC-AdCCL21 therapy. Significant reduction in tumor burden in a model in which tumors develop in an organ-specific manner provides a strong rationale for further evaluation of intrapulmonary-administered DC-AdCCL21 in regulation of tumor immunity and genetic immunotherapy for lung cancer. (Cancer Res 2006; 66(6): 3205-13)

Introduction

One of the challenges in developing immunotherapy for cancer is enlisting the host response to recognize poorly immunogenic tumors. Effective antitumor responses require antigen-presenting cells (APC), lymphocytes, and natural killer (NK) effectors (1–3). Although lung cancer cells express tumor antigens, limited expression of MHC antigens, defective transporters associated with antigen processing, and lack of costimulatory molecules make

them ineffective APC (4). In addition, tumor cells produce immune inhibitory factors that promote escape from immune surveillance (5). Tumor-reactive T cells accumulate in lung tumor microenvironment but fail to respond because of suppressive tumor cell-derived factors (6, 7). Additionally, a high proportion tumor-infiltrating lymphocytes in the tumor microenvironment are regulatory T cells (8).

Effective immune therapeutic strategies for lung cancer require methods to restore deficits in tumor antigen presentation and functional antitumor effector activities (9). Dendritic cells are bone marrow-derived leukocytes characterized by a high level of expression of MHC and costimulatory molecules. Their capacity to take up, process, and present antigens coupled with their ability to facilitate activation and expansion of antigen-specific T cells make them an attractive target for exploitation in designing strategies for cancer immunotherapy (10, 11). The use of dendritic cells pulsed with tumor antigen peptides, apoptotic tumor cells, tumor lysates, gene-encoding tumor antigens, and total tumor cell RNA has resulted in significant reductions of tumor burden in a variety of experimental paradigms (12–17). Thus, when appropriately armed with a tumor antigen, dendritic cells can promote antitumor immunity and significant tumor regression in lymphoma, renal cell carcinoma, and melanoma (18, 19).

Our approach tests the hypothesis that the use of chemokines to attract dendritic cells, lymphocytes, NK cell, and NK T-cell (NKT) effectors to the tumor site will be an effective treatment strategy. In addition, to overcome tumor microenvironment-associated suppressive effects on the dendritic cells, we have used a strategy that incorporates *ex vivo*-activated dendritic cells as the delivery vehicle for regional chemokine expression. Thus, we have transduced the gene encoding the CCR7 receptor ligand CCL21 (secondary lymphoid chemokine) into dendritic cells *ex vivo* and delivered the gene-modified dendritic cells (DC-AdCCL21) that secrete CCL21 into the tumor microenvironment. The introduction of dendritic cells at the tumor site provides immunization with the entire repertoire of available antigens *in situ*, increasing the likelihood of a response and reducing the potential for tumor resistance due to phenotypic modulation. CCL21 secretion by DC-AdCCL21 delivered to the tumor microenvironment also serves to recruit endogenous dendritic cells, T, NK, and NKT cells (20, 21). The recruitment of NK and NKT cells is advantageous because these effectors can recognize tumor targets in the absence of MHC expression (2, 3). Furthermore, CCL21 has potent angiostatic effects, providing additional rationale for use in cancer therapy (22).

Using s.c. murine lung cancer models, we have shown previously that intratumoral administration of cytokine gene-modified

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dendritic cells is more effective than equivalent concentrations of recombinant cytokines (9, 23). CCL21-transduced fibroblasts were not as effective as DC-AdCCL21, indicating that CCL21 is required for optimal antitumor responses and must be secreted by the dendritic cells for effective therapy. In this study, utilizing transgenic mice that develop lung cancer spontaneously, we show for the first time that intrapulmonary administration of CCL21 gene-modified dendritic cells (DC-AdCCL21) mediates effective anti-tumor responses *in vivo*, leading to a significant reduction in tumor burden and prolonged survival.

Materials and Methods

Reagents. The antibody pairs to murine IFN- γ , granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-10, and recombinant standards for these cytokines were from PharMingen (San Diego, CA). The antibody pairs to murine CXCL9, CXCL10, and transforming growth factor- β (TGF- β), and recombinant cytokine standards were purchased from R&D Systems, Inc. (Minneapolis, MN). Antimurine monoclonal antibody for CCL21 and recombinant CCL21 were purchased from PeproTech (Rocky Hill, NJ). Biotinylated antimurine antibody for CCL21 was obtained from R&D Systems. IL-12 determination was done with a kit from Biosource International (Camarillo, CA) according to the manufacturer's instructions. Prostaglandin E₂ (PGE₂) kit was obtained from Cayman Chemical (Ann Arbor, MI). Quantitative enzyme-linked immunospot (ELISPOT) for IFN- γ was done using a kit from PharMingen.

CC-10 T antigen mice. The transgenic CC-10 T antigen (TAG) mice, in which the SV40 large TAG is expressed under control of the murine Clara cell-specific promoter, were used in these studies (24). All of the mice expressing the transgene developed diffuse bilateral bronchoalveolar carcinoma in the lung. Tumors were evident bilaterally by microscopic examination as early as 4 weeks old. After age 3 months, the bronchoalveolar pattern of tumor growth coalesced to form multiple bilateral tumor nodules. The CC-10 TAG transgenic mice had an average life span of 4 months. Extrathoracic metastases were not noted. Breeding pairs for these mice were generously provided by Francesco J. DeMayo (Baylor College of Medicine, Houston, TX). Transgenic mice were bred at the West Los Angeles Veteran Affairs vivarium and maintained in the West Los Angeles Veterans Administration Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Research Facility. Before each experiment using the CC-10 TAG transgenic mice, presence of the transgene was confirmed by PCR of mouse-tail biopsies as described previously (25). All of the experiments used pathogen-free CC-10 TAG transgenic mice beginning at 5 to 6 weeks old. Mice were sacrificed when they showed signs of distress that included labored respiration and loss of appetite as indicated by a 10% loss in body weight.

Isolation and propagation of bone marrow-derived dendritic cells. Dendritic cells were isolated from bone marrow and incubated with lymphocyte- and macrophage-depleting antibodies (CD45R, anti-B-cell; TIB 229, anti-Ia; TIB 150, anti-CD8; and TIB 207, anti-CD4; all were obtained from the American Type Culture Collection, Manassas, VA) and rabbit serum complement (Sigma, St. Louis, MO) for 1 hour. Cells were washed and incubated overnight to allow contaminating macrophages to adhere, and nonadherent dendritic cells were harvested and cultured *in vitro* for 6 days with murine GM-CSF (2 ng/mL) and IL-4 (20 ng/mL; R&D Systems) as described previously (13). Consistent with previous studies from our laboratory as well as others (12, 13, 26), dendritic cells characterized by flow cytometry were found to have high-level expression of CD80, CD86, CD11c⁺DEC205⁺, MHC II, and MHC I. These cells were found to be 90% dendritic cells as defined by coexpression of these cell surface antigens (data not shown).

Preparation of adenoviral vectors and transduction of dendritic cells. The adenoviral construct (AdCCL21) is an E1-deleted, replication-deficient adenoviral type 5 vector encoding a 456-bp murine CCL21 cDNA. The control vector (AdRR5) did not contain the CCL21 cDNA insert. The AdCCL21 and AdRR5 adenoviral vectors were prepared as described

previously (23). The titer of each viral stock was routinely 10¹¹ to 10¹³ plaque-forming units (pfu) by plaque assay on 293 cells. Contamination with wild-type recombinant adenovirus was assessed for each viral stock by plaque assay on HeLa cells and was consistently negative. To optimize the multiplicity of infection (MOI) for CCL21 production, *in vitro*-propagated dendritic cells were transduced on day 7 in RPMI 1640 containing 2% fetal bovine serum (FBS) for 2 hours with AdCCL21 at MOIs of 10:1, 20:1, 50:1, and 100:1 in a 0.1 mL volume. For *in vivo* use in the murine lung cancer model, day 7 cultured dendritic cells were resuspended at a concentration of 1 × 10⁷/mL in RPMI 1640 containing 2% FBS and transduced for 2 hours with AdCCL21 at MOI of 100:1. The viral/cell suspension was mixed well. The transduced dendritic cells produced 7 to 10 ng CCL21 per 10⁶ cells checked by CCL21-specific ELISA as described previously (27), and transduced dendritic cells produced CCL21 for up to 17 days in culture. Using an adenovirus encoding green fluorescent protein (GFP) at a MOI of 100:1, we found that the transduction efficiency was ~70% (data not shown).

Therapeutic model in CC-10 TAG mice. Beginning at 3 months old, CC-10 TAG transgenic mice were injected once by intratracheal administration in 25 μ L volume with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) empty control adenoviral vector-transduced dendritic cells (DC-AdCV), (d) unmodified dendritic cells, (e) AdCCL21 (10⁸ pfu), and (f) AdCV (10⁸ pfu). Mice were anesthetized with ketamine/xylazine dose of 80 to 100/5 to 10 mg/kg by i.p. injection before administration of the various treatments. For groups receiving dendritic cells, 1 × 10⁶ dendritic cells were given. At 4 months, mice were sacrificed and lungs were isolated for quantification of tumor surface area. Tumor burden was assessed by microscopic examination of H&E-stained sections with a calibrated graticule (a 1-cm² grid subdivided into one hundred 1-mm² squares). A grid square with tumor occupying >50% of its area was scored as positive and the total number of positive squares was determined as described previously (28). Ten separate fields from four histologic sections of the lungs from six mice per group were examined under high power (objective, ×20). To assess time-based extent of mononuclear cell infiltration after treatment, CC-10 TAG transgenic mice were sacrificed at weekly intervals after therapy, and lungs were processed for histologic evaluation. To determine the distribution of the virus in the lung, we administered a replication-deficient AdGFP virus (10⁸ pfu) via intratracheal injection to anesthetized mice and evaluated GFP in the ornithine carbamyl transferase (OCT)-embedded fresh frozen lung microsections after 48 hours. To determine the effect of multiple treatments on survival benefit, mice were lightly anesthetized, and 1 × 10⁶ DC-AdCCL21 in 10 μ L volume with a pipette tip was given intranasally at weekly intervals for 8 weeks (*n* = 8 mice). The intranasal route was less traumatic than the intratracheal delivery and allowed for multiple administrations.

Flow cytometry. To quantify the phenotype of the mononuclear infiltrates following therapy, flow cytometric analyses for T-cell and dendritic cell markers were done on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) at the University of California at Los Angeles, Jonsson Cancer Center Flow Cytometry Core Facility. Two weeks following intratracheal instillation with DC-AdCCL21, lung tumors were harvested, cut into small pieces in RPMI 1640, and passed through a sieve (Bellco Glass, Vineland, NJ). Tumor leukocytes were isolated by digesting tumor tissue in collagenase IV (Sigma) in RPMI 1640 for 30 minutes with stirring at 37°C. A 10-mL syringe with a blunt-ended 16-gauge needle was used to break down the tissue further. The cell suspension was strained through a disposable plastic strainer (Fisher, Pittsburgh, PA) to separate free leukocytes from tissue matrix. The cells were pelleted at 2,000 rpm for 10 minutes, and cell pellets were washed twice to remove collagenase. Leukocytes were further purified using a discontinuous Percoll (Sigma) gradient, collecting at the 35% to 60% interface following centrifugation at 1,500 rpm for 20 minutes at 4°C without brake. The collected cells were washed twice in PBS and stained for flow cytometric evaluation. Following Percoll purification, the percentage of leukocytes in the cell population was >95%. Cells were identified as lymphocytes or dendritic cells by gating based on forward and side scatter profiles. CD11c⁺ dendritic cells were defined as the bright populations within tumor nodules. Ten thousand-gated events

were collected and analyzed using CellQuest software (Becton Dickinson). For staining, two or three fluorochromes (phycoerythrin, FITC, and peridinin chlorophyll protein; PharMingen) were used to gate on the CD4 and CD8 T lymphocytes or CD11c⁺DEC205⁺ dendritic cells in purified single-cell leukocyte populations from the tumor nodules.

Cytokine-specific ELISA. Three-month-old CC-10 TAG transgenic mice were treated with the various therapies stated above, and at 4 months, cytokine protein concentrations from tumor nodules and spleens were determined by ELISA as described previously (13). Lungs were harvested, cut into small pieces, homogenized, and passed through a sieve (Belco Glass). Spleens were harvested and teased apart, and RBC were depleted with double-distilled H₂O. Cytokines (GM-CSF, IFN- γ , IL-10, IL-12, CXCL9, CXCL10, and TGF- β) were determined by ELISA in homogenized tumors directly and splenocytes (5×10^6 cells/mL) following a 24-hour culture. For the TGF- β ELISA measurements, samples were acidified; hence, the active form of TGF- β was measured. Tumor-derived cytokine concentrations were corrected for total protein by Bradford assay (Sigma), and the results were expressed as pg/mg total protein. The sensitivities of the IL-10, GM-CSF, IFN- γ , TGF- β , IL-12, CXCL9, and CXCL10 ELISA were 15 pg/mL. The plates were read at 490 nm with a microplate reader (Molecular Devices Corp., Sunnyvale, CA).

PGE₂ enzyme immunoassay. PGE₂ concentrations were determined using a kit from Cayman Chemical according to the manufacturer's instructions as described previously (5). The enzyme immunoassay plates were read by a microplate reader (Molecular Dynamics Corp., Sunnyvale, CA).

IFN- γ ELISPOT. To evaluate the immune specificity of the treatments, IFN- γ ELISPOT assay was done to determine the frequency of T lymphocytes producing IFN- γ in response to specific tumors. On day 14 posttreatment of 3-month-old mice, splenic T lymphocytes were purified by negative selection using Miltenyi Biotec (Auburn, CA) beads. T lymphocytes were coincubated with either irradiated specific CC-10 cell line or nonspecific syngeneic MLE-12 cell lines at a lymphocyte effectors-to-stimulator ratio of 10:1 for 24 hours. A single-cell suspension of CC-10 or MLE-12 tumor cells (10^6 /mL) was irradiated with 80 Gy γ -irradiation in a ¹³⁷Cs γ -irradiator. Spots were quantified with an Immunospot Image Analyzer (Cellular Technologies Ltd., Cleveland, OH) at the University of California at Los Angeles Immunology Core Facility.

In vitro cytotoxicity. Innate and specific antitumor responses were evaluated following therapy. Three-month-old CC-10 TAG transgenic mice were treated with 1×10^6 DC-AdCCL21 or diluent intranasally at weekly intervals for 3 weeks. One week following the last treatment, NK and T cells were purified from spleens by negative selection using Miltenyi Biotec beads, and cytolytic activities were evaluated against autologous CC-10 tumor cell line and syngeneic MLE-12 cell line. The NK and T-cell effectors were incubated with tumor cell targets (E:T of 32:1 and 64:1) in quadruplet wells in a 96-well plate, and 20 μ L Alamar Blue (Cellular Technologies Ltd., Camarillo, CA) was added to each well after 18 hours of incubation. The plate was read with the Wallac 1420 fluorescence plate reader (Perkin-Elmer Life Science, Turku, Finland) with the excitation/emission set at 530/590 nm.

Intracellular staining for large TAG. Single-cell suspensions of CC-10 and MLE-12 cells were stained for the large TAG using the BD cytofix/cytoperm (BD Biosciences, San Diego, CA) solution according to the manufacturer's instructions. Briefly, cells were fixed and permeabilized in cytofix/cytoperm solution for 30 minutes. The cell pellet was resuspended in 100 μ L Perm/Wash and stained with 0.5 μ g purified mouse anti-SV40 large TAG for 30 minutes. Cells were washed twice in Perm/Wash buffer and stained with FITC-labeled sheep anti-mouse IgG for 30 minutes. Cells were suspended in 300 μ L PBS/2% paraformaldehyde solution and analyzed by flow cytometry. Controls included cells stained with the secondary antibody alone from nonspecific staining, and 3LL-stained cells served as the negative control.

Statistical analyses. Groups of 8 to 10 mice were used. Statistical analyses of the data were done using the Kruskal-Wallis one-way ANOVA on ranks followed by multiple pair-wise comparisons according to Dunn's method. Significance at the $P < 0.05$ level is denoted.

Results

Intrapulmonary administration of DC-AdCCL21 reduces tumor burden in a model of spontaneous lung cancer. We evaluated the antitumor efficacy of DC-AdCCL21 in a spontaneous bronchoalveolar cell carcinoma model in transgenic mice, in which the SV40 large TAG is expressed under control of the murine Clara cell-specific promoter, CC-10 (24). Mice expressing the transgene develop diffuse bilateral bronchoalveolar carcinoma, which eventuate in respiratory failure and death at ~ 4 months. Beginning at a time when mice typically exhibit a substantial pulmonary tumor burden (3 months old), CC-10 TAG transgenic mice were injected intratracheally with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) DC-AdCV, (d) unmodified dendritic cells, (e) AdCCL21 (10^8 pfu), and (f) AdCV (10^8 pfu). At 4 months when control mice started to succumb because of progressive lung tumor growth, mice in all of the treatment groups were sacrificed, lungs were isolated, and tumor burdens were quantified. Control mice exhibited large tumor masses throughout both lungs with minimal mononuclear cell infiltration (Fig. 1A and B), whereas there was a decrease in the tumor burden in AdCCL21 (1.3-fold) and DC-AdCCL21 (1.9-fold; $P < 0.001$, compared with diluent control) treatment groups (Fig. 1D). The AdCV and DC-AdCV had marginal (not significant) decreases in tumor burden, suggesting that the reported antiviral CTL responses to E1-deleted adenoviral vectors (29) were not responsible for reducing tumor burden in the CC-10 TAG mice. In contrast, mice treated once with either AdCCL21 or DC-AdCCL21 had a significant reduction in pulmonary tumor burden compared with diluent and the other treatment groups (Fig. 1A and D). The reduction in tumor burden was enhanced in the DC-AdCCL21 treatment group compared with AdCCL21 treatment ($P < 0.001$). This finding suggests that CCL21 secretion via adenovirus-mediated transduction of tumor and stromal cells *in situ* within the tumor microenvironment does not yield as optimal an antitumor response as is mediated by DC-AdCCL21. These findings suggest that both dendritic cells and CCL21 secreted by the transduced dendritic cells may be required for effective therapy in this model system. Whereas diluent-treated control mice revealed large tumor masses throughout both lungs with minimal mononuclear cell infiltration, the small residual tumor nodules in DC-AdCCL21-treated mice had extensive infiltration. The mononuclear cell infiltration was evident within 1 week of a single intratracheal dose of DC-AdCCL21 but was most pronounced 2 weeks following treatment (Fig. 1B). Flow cytometric analyses of the mononuclear cell infiltrates showed significant increase in CD4 (1.9-fold), CD8 (1.7-fold), and CD11c⁺DEC205⁺ dendritic cells (2.3-fold) compared with diluent-treated controls ($P < 0.001$; Fig. 1C). DC-AdCCL21 treatment prolonged median survival (17 ± 1 weeks for control mice and $>20 \pm 1$ weeks for mice treated with DC-AdCCL21; $P < 0.001$). All other therapies (dendritic cells alone, AdCV, and DC-AdCV) resulted in a median survival of 18 ± 1 weeks. To determine the distribution of the adenovirus in the lung of CC-10 transgenic mice, a replication-deficient AdGFP virus (10^8 pfu) was administered via intratracheal injection to anesthetized mice and GFP in the OCT-embedded fresh frozen lung microsections evaluated after 48 hours. GFP was detected "regionally" in the terminal airway (data not shown). Administering DC-AdCCL21 in a continuous dosing regimen at weekly intervals for 8 weeks further enhanced the survival benefit. In this paradigm, the median survival increased to 24 ± 1 weeks ($P < 0.001$; Fig. 2).

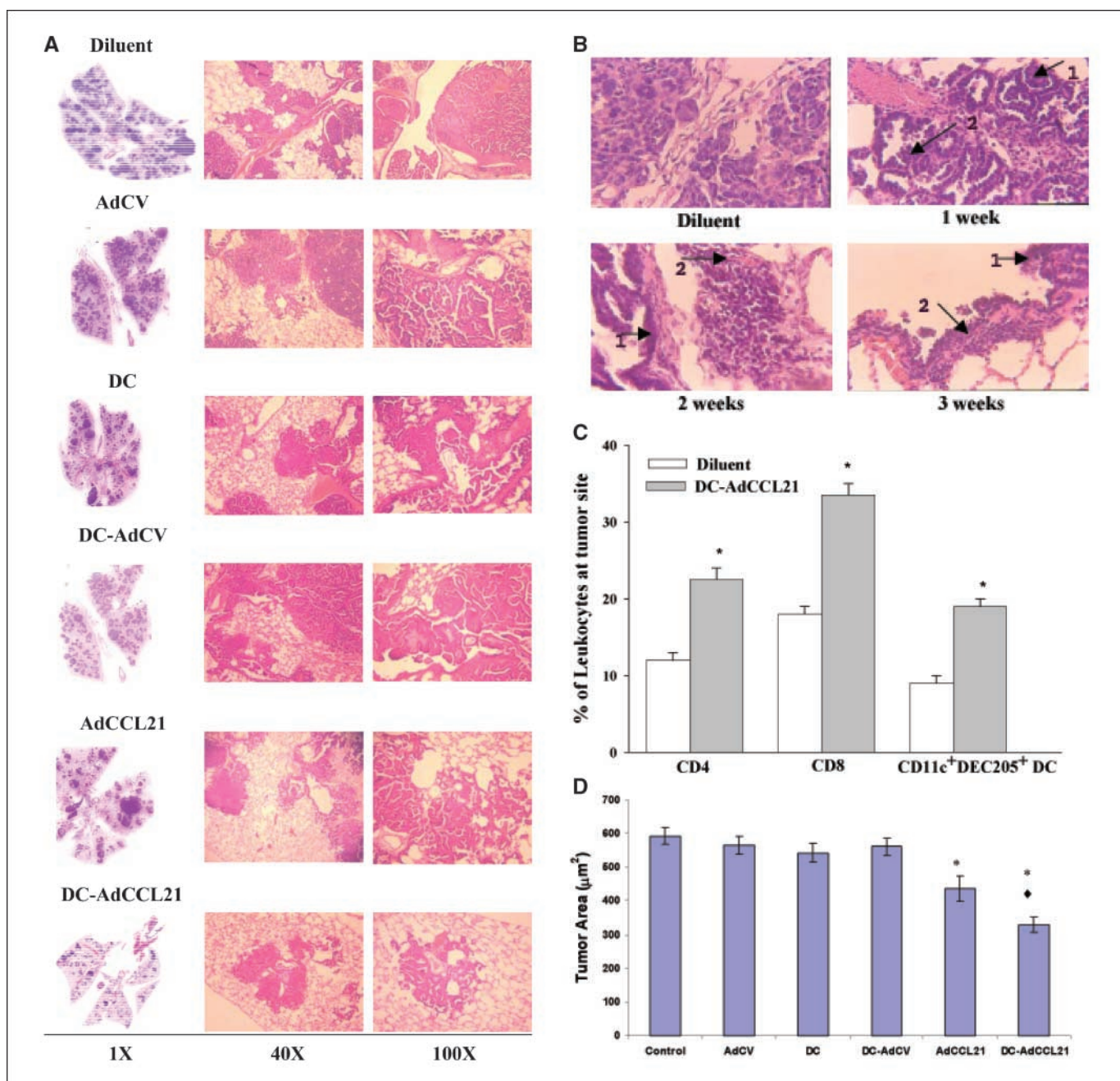


Figure 1. Intrapulmonary administration of DC-AdCCL21 mediates potent antitumor responses in a murine model of spontaneous lung cancer. Beginning at 3 months old, CC-10 TAg mice were injected once intratracheally with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) DC-AdCV, (d) unmodified dendritic cells, (e) AdCCL21 (10^8 pfu), and (f) AdCV (10^8 pfu). *A*, H&E staining of paraffin-embedded lung tumor sections from control-treated mice evidenced large tumor masses throughout both lungs without detectable mononuclear infiltration. Compared with controls, there was a decrease in the tumor burden in the AdCCL21 and DC-AdCCL21 treatment groups. However, compared with all treatment groups, DC-AdCCL21 treatment group evidenced extensive mononuclear infiltration with marked reduction in tumor burden. Magnifications, $\times 1$, $\times 40$, and $\times 100$. *B*, mononuclear cell infiltration (1, tumor; 2, leukocytes) depicted at 1, 2, and 3 weeks after DC-AdCCL21 therapy. Magnification, $\times 400$. *C*, compared with diluent-treated control, there was an increase in CD4, CD8, and CD11c⁺DEC205⁺ dendritic cell infiltrates in the DC-AdCCL21 treatment group. *, $P < 0.001$. *D*, tumor burden was quantified within the lung by microscopy of H&E-stained paraffin-embedded sections. DC-AdCCL21 treatment led to the greatest reduction in tumor burden compared with all other treatment groups. Bars, SE. *, $P < 0.001$ compared with diluent-treated control; ♦, $P < 0.01$ compared with AdCCL21 treatment group ($n = 8-10$ mice per group).

DC-AdCCL21 therapy promotes type 1 cytokine and antiangiogenic chemokine release but a decline in the immunosuppressive molecules TGF- β , IL-10, and PGE₂ in CC-10 TAg mice. Based on previous reports indicating that tumor progression can be modified by host cytokine profiles (30, 31), we measured the cytokine production from tumor sites and spleens following

therapy. Lungs and spleens were evaluated in the presence of IL-10, IFN- γ , GM-CSF, IL-12, CXCL9, CXCL10, and TGF- β by ELISA and PGE₂ by enzyme immunoassay. Compared with diluent-treated controls, the treatment group receiving AdCCL21 had a modest but significant increase in type 1 cytokines (IFN- γ and IL-12) and antiangiogenic chemokines (CXCL9 and CXCL10) and

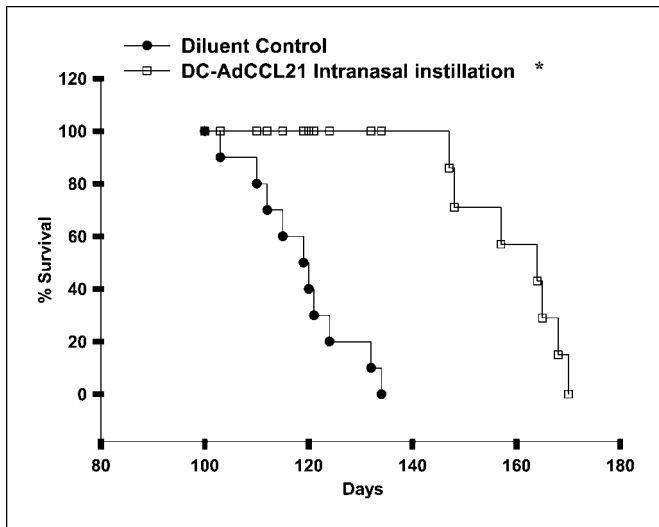


Figure 2. DC-AdCCL21 administration prolongs survival in CC-10 TAG mice. Beginning at 3 months old, CC-10 TAG mice were treated intranasally once weekly for 8 weeks with diluent or DC-AdCCL21, and survival was monitored. Survival was significantly prolonged in the DC-AdCCL21 treatment group. *, $P < 0.001$ compared with diluent-treated control.

a decrease in the immunosuppressive mediators (PGE₂ and TGF-β) at the tumor sites. However, as was evident for tumor reduction, the DC-AdCCL21-treated group produced the most impressive increases in type 1 cytokines and antiangiogenic chemokines as well as the most substantial decline in the pulmonary production of immunosuppressive mediators in the tumor microenvironment. Compared with lungs from the diluent-treated group, CC-10 TAG mice treated with DC-AdCCL21

had significant reductions in IL-10 (1.5-fold; $P < 0.01$), PGE₂ (1.8-fold; $P < 0.01$), and TGF-β (2.5-fold; $P < 0.05$). This was coupled with an increase in GM-CSF (2.3-fold; $P < 0.01$), IFN-γ (2.6-fold; $P < 0.01$), CXCL9 (1.4-fold; $P < 0.01$), CXCL10 (2-fold; $P < 0.05$), and IL-12 (4-fold; $P < 0.05$) within the tumor microenvironment (Fig. 3A and B). Moreover, a systemic effect was evident, as similar cytokine patterns were also observed in the spleens of DC-AdCCL21-treated mice. Thus, compared with the diluent-treated group, splenocytes from DC-AdCCL21-treated CC-10 TAG mice revealed reduced levels of PGE₂ (2.4-fold; $P < 0.05$) and TGF-β (2.1-fold; $P < 0.05$) but an increase in GM-CSF (3-fold; $P < 0.01$), IFN-γ (16-fold; $P < 0.001$), CXCL9 (5-fold; $P < 0.05$), CXCL10 (2-fold; $P < 0.05$), and IL-12 (3-fold; $P < 0.05$; Fig. 4A and B).

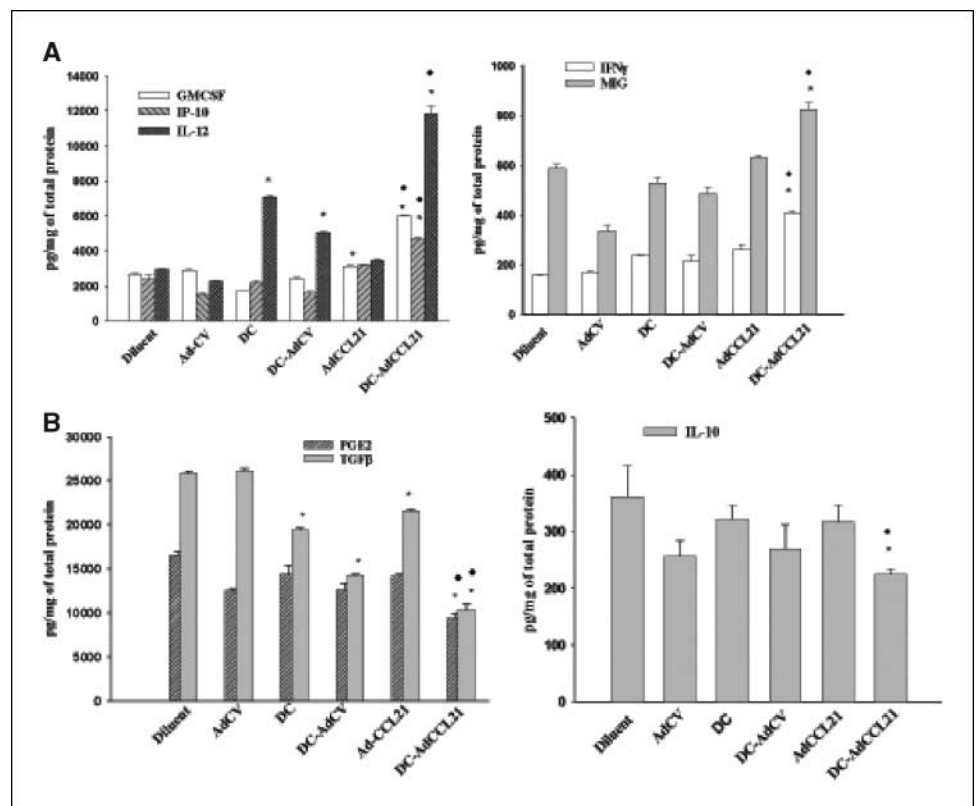
DC-AdCCL21 therapy induces specific T-cell responses.

To evaluate the induction of tumor-specific T cells, IFN-γ ELISPOT assays were done. Compared with diluent-treated control and other therapies, DC-AdCCL21 group had significantly greater frequency (20-fold) of specific T cells releasing IFN-γ when restimulated with irradiated CC-10 cells ($P < 0.001$). The minimal responses to the syngeneic control tumor MLE-12 may be due to shared antigens because MLE-12 is also derived from a SV40 large TAG-induced spontaneous tumor in the FVB mice (Fig. 5).

Both innate and specific antitumor responses were enhanced following therapy.

Three-month-old CC-10 TAG transgenic mice were given diluent or DC-AdCCL21 intranasally once weekly for 3 weeks. One week following the last treatment, NK and T cells were purified from spleens, and their cytolytic activities were evaluated against the autologous CC-10 tumor cell line. Compared with diluent-treated mice, the DC-AdCCL21 group had significantly greater NK (3- to 5-fold) and T cell (2- to 5-fold)

Figure 3. A and B, DC-AdCCL21 therapy induces type 1 cytokines and antiangiogenic chemokines and a decrease in immunosuppressive molecules in the lung tumor milieu of CC-10 TAG mice. Beginning at 3 months old, CC-10 TAG mice were injected once intratracheally with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) DC-AdCV, (d) unmodified dendritic cells, (e) AdCCL21 (10⁸ pfu), and (f) AdCV (10⁸ pfu). Cytokines (GM-CSF, IFN-γ, IL-10, IL-12, CXCL9, CXCL10, and TGF-β) were determined by ELISA and PGE₂ by enzyme immunoassay in homogenized tumors. Tumor-derived cytokine concentrations were corrected for total protein by Bradford assay. Results were expressed as pg/mg total protein. Compared with tumor nodules from control group, mice treated with DC-AdCCL21 had significant increase in GM-CSF, IFN-γ, CXCL9, CXCL10, and IL-12 but a decrease in the immunosuppressive molecules PGE₂, IL-10, and TGF-β. Bars, SE. *, $P < 0.01$ compared with diluent-treated control; ♦, $P < 0.01$ compared with other treatment groups ($n = 8$ mice per group).



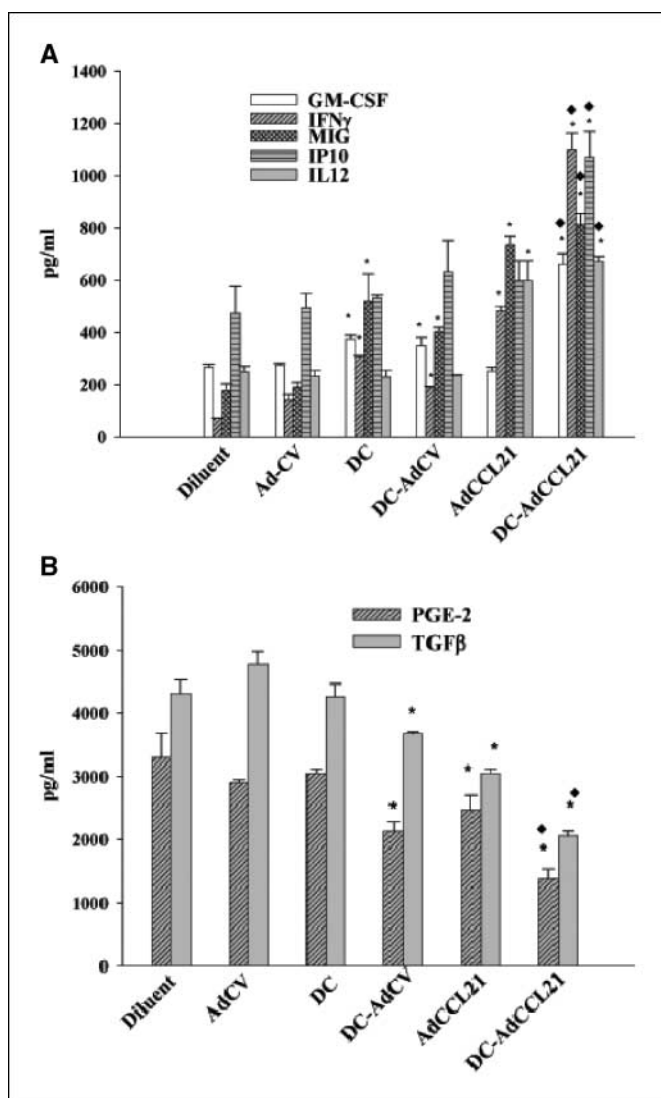


Figure 4. A and B, DC-AdCCL21 therapy promotes type 1 cytokines and antiangiogenic chemokines but a decrease in immunosuppressive molecules systemically of CC-10 TAG mice. Beginning at 3 months old, CC-10 TAG mice were injected once intratracheally with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) DC-AdCV, (d) unmodified dendritic cells, (e) AdCCL21 (10^8 pfu), and (f) AdCV (10^8 pfu). Cytokines (GM-CSF, IFN- γ , IL-10, IL-12, CXCL9, CXCL10, and TGF- β) were determined by ELISA and PGE $_2$ by enzyme immunoassay in splenocyte supernatants (5×10^6 cells/mL) after a 24-hour culture. Results were expressed as pg/mL. Bars, SE. *, $P < 0.01$ compared with diluent-treated control; \diamond , $P < 0.01$ compared with other treatment groups ($n = 8$ mice per group).

capacity to lyse CC-10 tumors *in vitro* ($P < 0.001$). In the DC-AdCCL21 treatment group, the T-lytic cell response against the CC-10 tumor cells was greater than the syngeneic control tumor MLE-12 ($P < 0.001$). NK cell-mediated lysis of MLE-12 cells was enhanced to similar levels as CC-10 cells following DC-AdCCL21-mediated therapy (Fig. 6).

Discussion

In an attempt to stimulate specific antitumor immunity, experimental models and clinical studies are currently evaluating the potent antigen-presenting capacity of dendritic cells combined with single or multiple tumor antigen epitopes (32). However, the

problems in using tumor antigen-based immunization strategies include (a) the potential induction of tolerance (33), (b) the inability to use repeated dosing because of vector-associated neutralization (34), and (c) the limitation of therapy to patients whose tumors express defined specific tumor antigens in the context of the correct HLA phenotype (35).

We and others have described previously a therapeutic paradigm that overcomes these deficits by intratumoral administration of cytokine gene-modified dendritic cells (9, 23, 36). This antitumor dendritic cell-based therapy exploits the professional APC as an effective vehicle for cytokine delivery and presentation of multiple tumor antigens *in situ*. In recent studies, we have shown that intratumoral administration of dendritic cells overexpressing CCL21 generates systemic antitumor responses and confers tumor immunity (23). In these studies, CCL21 secreted by dendritic cells constituted a critical component for the generation of IFN- γ , CXCL9, and CXCL10 effector molecules that were responsible for the antitumor response.

However, in the models reported previously, the antitumor efficacy of DC-AdCCL21 was determined using transplantable murine or human tumors propagated at s.c. sites. Thus, we embarked on the current studies to determine the antitumor properties of DC-AdCCL21 in a relevant model of lung cancer, in which adenocarcinomas develop spontaneously in an organ-specific manner and represent a proximate model of human lung cancer. Based on our observations that CCL21 gene-modified dendritic cells have enhanced secretion of IP-10, MIG, and IL-12 that are known to have potent antitumor properties, we hypothesized that the intrapulmonary delivery of DC-AdCCL21

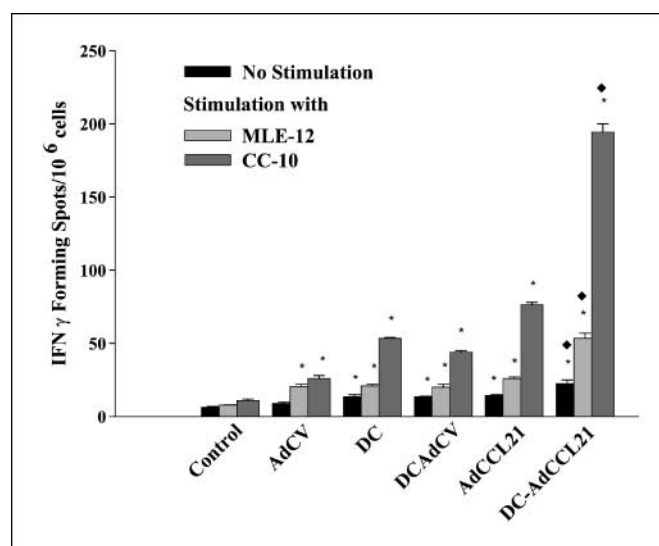


Figure 5. DC-AdCCL21 therapy induces specific T-cell response. Beginning at 3 months old, CC-10 TAG mice were injected once intratracheally with one of the following treatments: (a) diluent, (b) DC-AdCCL21, (c) DC-AdCV, (d) unmodified dendritic cells, (e) AdCCL21 (10^8 pfu), and (f) AdCV (10^8 pfu). Two weeks following therapy, antigen-specific T-cell response was determined by ELISPOT. Spleen T lymphocytes were restimulated overnight with irradiated CC-10 and nonspecific MLE-12 tumors at a ratio of 10:1. Mouse IFN- γ -specific ELISPOT was done, and spots were quantified with an Immunospot Image Analyzer. Compared with diluent-treated control and other therapies, T lymphocytes from DC-AdCCL21-treated group had significantly greater frequency of specific T cells releasing IFN- γ when restimulated with CC-10 TAG cells. There were minimal responses to the syngeneic control tumor MLE-12. Bars, SE. *, $P < 0.001$ compared with diluent-treated group; \diamond , $P < 0.001$ compared with other treatment groups ($n = 8$ mice per group).

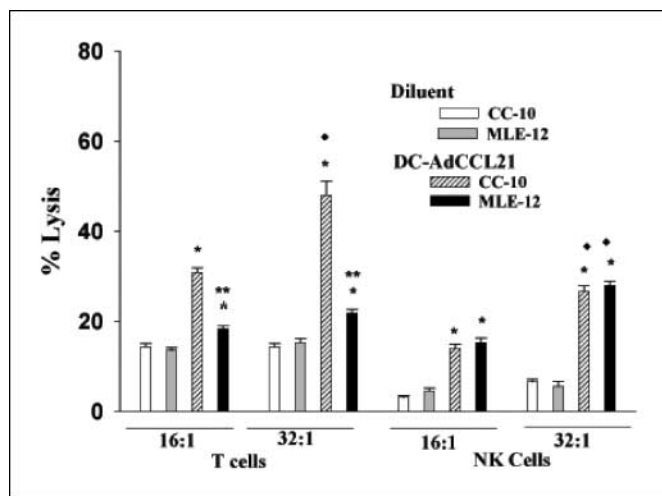


Figure 6. Both innate and specific antitumor responses were enhanced following therapy. Three-month-old CC-10 TAG transgenic mice were given intranasally (a) diluent or (b) DC-AdCCL21 once weekly for duration of 3 weeks. One week following the last treatment, NK and T cells were purified from spleens, and their cytolytic activities were evaluated against autologous CC-10 and syngeneic MLE-12 tumor cell lines at E:T of 32:1 and 64:1. Compared with diluent-treated control, NK and T cells from DC-AdCCL21 group had significantly greater capacity to lyse autologous CC-10 tumors *in vitro*. *, $P < 0.001$. In the DC-AdCCL21-treated group, the T-lytic cell response against the CC-10 tumor cells was greater than the syngeneic control tumor MLE-12. **, $P < 0.001$. Columns, mean; bars, SE. ♦, $P < 0.001$ compared with E:T of 16:1 ($n = 6$ mice per group).

in the CC-10 TAG mice would lead to effective antitumor responses. In this study, CC-10 TAG mice were treated using a novel mode of gene-modified cell delivery. Specifically, gene-modified dendritic cells were not given directly into the tumor compartment (as was accomplished with intratumoral injection in the s.c. model), but rather the cellular therapy was delivered regionally within the vicinity of the tumor. Thus, animals were given DC-AdCCL21 once intratracheally and subsequently (in the continued administration method) through nasal airways. This mode of delivery was used to mimic the clinical setting of lung cancer, whereby cellular immunotherapy would be delivered by means of fiberoptic bronchoscopy. The results suggest that both dendritic cells and CCL21 secreted by the transduced dendritic cells may be required to optimize the antitumor response but also indicate that significant antitumor responses can be generated with a single regional instillation of DC-AdCCL21. Our results show that a single intratracheal instillation of DC-AdCCL21 mediate effective antitumor responses, tumor reduction, and survival advantage of 3 weeks compared with diluent-treated control therapy. To determine the distribution of the replication-defective adenovirus, we administered a replication-deficient AdGFP virus (10^8 pfu) via intratracheal injection and detected expression of GFP regionally in the terminal airway. Moreover, weekly intranasal instillations further enhanced the effectiveness of the approach by extending the median survival by 7 weeks. This observation provided evidence that intrapulmonary delivery of DC-AdCCL21 was able to mediate antitumor responses.

The effectiveness of this approach is likely attributable to the cascade of events that is initiated within the tumor microenvironment by delivery of DC-AdCCL21. At the outset, there is a local elaboration of type 1 cytokines and a concomitant decline in immunosuppressive factors within the milieu. Within 1 week after

local administration of DC-AdCCL21, there is a mononuclear influx into the tumor with increases in $CD4^+$ and $CD8^+$ T cells and $CD11c^+DEC205^+$ dendritic cells. Parallel *in vitro* cytolytic assessments suggest that both T and NK effector arms may be effective in mediating tumor cell lysis following intranasal (regional) instillation of DC-AdCCL21 in CC-10 TAG mice. Although both CC-10 and MLE-12 cell lines express similar levels of the SV40 large TAG (data not shown), the results from the *in vitro* cytotoxicity experiment in Fig. 6 suggest that responses to the large TAG alone cannot explain antitumor specific T-cell responses. Had the T-cell responses only been to the large TAG, we would expect similar level of lysis for both cell lines *in vitro*. In addition, for tumors to form in the CC-10 TAG transgenic mice, the T cells have to be tolerant to the SV40 large TAG. The innate enhanced NK responses, however, were the same for both cell lines. The results of this experiment suggest that both innate and antitumor T-cell responses were enhanced following therapy. The enhancement in NK tumor cytolytic activity is important because these effector cells can recognize tumor targets in the absence of MHC expression (2, 3). In future studies, we will quantify the contribution of T and NK cell effectors to the antitumor responses.

We found previously that specific enhanced T lymphocytes release of $IFN-\gamma$ and GM-CSF following treatment with DC-AdCCL21 (23). The specific cytokine release data from *in vitro* studies were consistent with the role of the DC-AdCCL21 in antitumor responses *in vivo* (23). Consistent with these findings, the cytokine production from tumor sites and spleens (IL-10, PGE_2 , $TGF-\beta$, $IFN-\gamma$, GM-CSF, CXCL9, CXCL10, and IL-12) in the CC-10 TAG mice was altered by DC-AdCCL21 therapy (Figs. 2 and 3). Before DC-AdCCL21 treatment in the transgenic tumor-bearing mice, the levels of the immunosuppressive proteins IL-10, PGE_2 , and $TGF-\beta$ were elevated when compared with the levels in normal control mice (data not shown). DC-AdCCL21-treated CC-10 TAG mice showed significant reductions in the immunosuppressive molecules IL-10, PGE_2 , and $TGF-\beta$. The decrease in immunosuppressive cytokines was not limited to the lung but was also evident systemically. Thus, possible benefits of a DC-AdCCL21-mediated decrease in these cytokines include promotion of antigen presentation and CTL generation (28, 37) as well as a limitation of angiogenesis (38, 39).

Successful immunotherapy shifts tumor-specific T-cell responses from a type 2 to a type 1 cytokine profile (40). Responses depend on IL-12 and $IFN-\gamma$ to mediate a range of biological effects, which facilitate anticancer immunity. The lungs of DC-AdCCL21-treated CC-10 TAG mice revealed significant increases in GM-CSF, $IFN-\gamma$, CXCL9, CXCL10, and IL-12. An increase in $IFN-\gamma$ at the tumor site of DC-AdCCL21-treated mice could explain the relative increases in CXCL9 and CXCL10. Both CXCL9 and CXCL10 are chemotactic for stimulated CXCR3-expressing T lymphocytes that could additionally amplify $IFN-\gamma$ at the tumor site (41). The increase in type 1 cytokines may in part be due to an increase in both $CD4^+$ and $CD8^+$ T-cell infiltrates as well as an increase in T-cell responses against autologous tumor. DC-AdCCL21-treated mice had a significantly increased frequency of T cells producing $IFN-\gamma$ in response to autologous tumor. Additional studies are necessary to precisely define the T-cell subsets and the host cytokines that are critical to the DC-AdCCL21-mediated antitumor response.

Host APC are critical for the cross-presentation of tumor antigens (1, 10). However, tumors have the capacity to limit APC

maturation, function, and infiltration of the tumor site (42–44). The current model system overcomes this detriment by activating dendritic cells precursors *ex vivo* in GM-CSF and IL-4. This allows dendritic cell propagation to occur in an environment conducive to full activation without interference from deleterious tumor-derived products. Thus, the use of activated dendritic cells secreting CCL21 may help recruit host dendritic cells that process and present tumor antigens to initiate and/or maintain antitumor immune responses. In light of this, recent work by Flanagan et al. showed that CCL21 costimulates naive T-cell expansion and Th1 polarization of nonregulatory CD4⁺ T cells (45). In other studies, CCL21 enhanced the immunity by a DNA melanoma vaccine (46) and the antitumor effect of the costimulatory molecule light (47) by greatly enhancing the tumor infiltration of mature dendritic cells and CD8⁺ T cells. CCL21 has also been identified as a potent maturation factor for dendritic cells and indirect regulator for Th1 cell differentiation (48). Hence, the antitumor properties of DC-AdCCL21 in our model may be attributable to the stimulation of host antigen-presenting functions by CCL21, increased

antiangiogenic activities mediated via IFN- γ induction of CXCL9 and CXCL10, and down-regulation in immunosuppressive molecules PGE₂, IL-10, and TGF- β . Additional studies will be required to delineate the importance of each of these cytokines in DC-AdCCL21-mediated antitumor responses, and the precise role of the dendritic cells in this model awaits definition. The potent antitumor properties shown in this model of spontaneous bronchoalveolar carcinoma provide a strong rationale for additional evaluation of DC-AdCCL21 in the regulation of tumor immunity.

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