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

Seok-Chul Yang,1,2,3 Raj K. Batra,1,2,3 Sven Hillinger,4 Karen L. Reckamp,1,2,3 Robert M. Strieter,1,2 Steven M. Dubinett,1,2 and Sherven Sharma1,2

1Department of Medicine, Lung Cancer Research Program and 2Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California at Los Angeles; Molecular Medicine Laboratory, Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California; and 3Thoracic Surgery, University Hospital Zurich, Zurich, Switzerland

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 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 transfected 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
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 antitumor 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 CXCL19, 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), as purchased from Cayman Chemical (Ann Arbor, MI). Quantitative enzyme-linked immunosorbent assay (ELISPOT) kit 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 Veterans Affairs vivarium and maintained in the West Los Angeles Veterans Administration Administration 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 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 immunosorbent assay (ELISPOT) kit for IFN-γ was done using a kit from PharMingen.

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 CC-121 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 μL 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 (105 pfu), and (f) AdCV (105 pfu). Mice were anesthetized with ketamine/xylazine dose of 80 to 100/5 to 10 mg/kg by ip. 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 HE-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 (105 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 × 105 DC-AdCCL21 in 10 μL 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-μL 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). The sensitivities of the IL-10, GM-CSF, IFN-γ, IL-12, CXC19, 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. Significance at the P<0.001 level 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 (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 ± 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).

**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 (10^5 pfu), and (f) AdCV (10^5 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 ± 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).
DC-AdCCL21 therapy promotes type 1 cytokine and anti-angiogenic 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...
had significant reductions in IL-10 (1.5-fold; $P < 0.01$), PGE$_2$ (1.8-fold; $P < 0.01$), and TGF-$\beta$ (2.5-fold; $P < 0.05$). This was coupled with an increase in GM-CSF (2.3-fold; $P < 0.01$), IFN-$\gamma$ (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 PGE$_2$ (2.4-fold; $P < 0.05$) and TGF-$\beta$ (2.1-fold; $P < 0.05$) but an increase in GM-CSF (3-fold; $P < 0.01$), IFN-$\gamma$ (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-$\gamma$ 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-$\gamma$ 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)
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-$\gamma$, 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
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 (10⁸ 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-γ 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, PGE₂, TGF-β, IFN-γ, 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, PGE₂, and TGF-β 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, PGE₂, and TGF-β. 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-γ 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-γ, CXCL9, CXCL10, and IL-12. An increase in IFN-γ 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-γ 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-γ 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 \textit{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 naïve 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.

Acknowledgments

Received 10/10/2005; revised 12/22/2005; accepted 1/10/2006.

Grant support: NIH grants R01 CA85686 and UCLA Lung Cancer SPORE P50 CA90388, Department of Veteran Affairs Medical Research Funds, Research Enhancement Award Program in Cancer Gene Medicine, and Tobacco-Related Disease Research Program of the University of California.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

24. Tsuji S, Kawano S, Tsuji M, et al. Mucosal...


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

Seok-Chul Yang, Raj K. Batra, Sven Hillinger, et al.


### Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/6/3205

### Cited articles
This article cites 48 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/6/3205.full#ref-list-1

### Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/66/6/3205.full#related-urls

### E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

### Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

### Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/66/6/3205.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.