Intratumoral Administration of Dendritic Cells Overexpressing CCL21 Generates Systemic Antitumor Responses and Confers Tumor Immunity

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ABSTRACT

To achieve in situ tumor antigen uptake and presenta-
tion, intratumoral administration of ex vivo-generated, gene-
modified murine bone marrow-derived dendritic cells (DC)
was used in a murine lung cancer model. To attract mature
host DC and activated T cells at the tumor site, the DC were
transduced with an adenoviral vector expressing secondary
lymphoid tissue chemokine (CCL21/SLC). Sixty percent of
the mice treated with 10^6 DC-AdCCL21 intratumorally
(7–10 ng/ml/10^6 cells/24 h of CCL21) at weekly intervals for
3 weeks showed complete tumor eradication, whereas only
25% of mice had complete resolution of tumors when mice
were treated with fibroblasts expressing CCL21. In contrast
only 12% of the mice treated with unmodified or control
vector modified DC (DC-AdCV) showed complete tumor
eradication. DC-AdCCL21 administration led to increases in
the CD4^+ , CD8^+ , and CD3^+ CXCR3^+ T cells, as well as
DC expressing CD11c^+ DEC205^-CD45^- T regulatory cells
infiltrating the tumors were markedly reduced after
DC-AdCCL21 therapy. The tumor site cellular infiltrates
were accompanied by the enhanced elaboration of

granulocyte macrophage colony-stimulating factor, IFN-γ,
MIG/CXCL9, IP-10/CXCL10, and interleukin 12, but de-
creases in the immunosuppressive mediators transforming
growth factor β and prostaglandin E_2. DC-AdCCL21-
treated tumor-bearing mice showed enhanced frequency of
tumor-specific T lymphocytes secreting IFN-γ, and tumor
protective immunity was induced after DC-AdCCL21 ther-
apy. In vivo depletion of IP-10/CXCL10, MIG/CXCL9, or
IFN-γ significantly reduced the antitumor efficacy of DC-
AdCCL21. These findings provide a strong rationale for the
evaluation of DC-AdCCL21 in cancer immunotherapy.

INTRODUCTION

Functional host professional antigen presenting cells
(APC) have been demonstrated to be of central importance
in the immune response (1). Dendritic cells (DC) are highly
specialized professional APCs with potent capacity to capture,
process, and present antigen to T cells (2). However, tumor cells
interfere with host DC maturation and function (3–5). To cir-
cumvent tumor-mediated inhibition of DC maturation and func-
tion, ex vivo cytokine-stimulated DCs have been effectively
used in clinical trials and murine cancer models (6–14). Anti-
gen-specific CTL responses can be achieved by using ex vivo
antigen-pulsed DCs (7, 15–18) or DCs transfected with tumor
RNA. Additionally, novel tumor antigen delivery systems using
cytokine gene-transduced tumor cells and DCs (6, 9), fusion
of tumor cells with DCs, and intratumoral injection of cytokine-
modified DCs have been effectively used to induce antitumor
immunity (19–21). Delivery of tumor antigens by ex vivo-
stimulated DCs has been shown to be superior to purified
peptides in avoiding CTL tolerance (22). Vaccination with mul-
tiple tumor antigens may be superior to the use of single
epitopes (23, 24), and these responses can be additionally en-
hanced by the coadministration of immune-potentiating cyto-
kines (6, 9, 25). Consequently, it has been suggested that effec-
tive anticancer immunity may be achieved by recruiting
professional host APCs for tumor antigen presentation to pro-
 mote specific T-cell activation (26). Thus chemokines that at-
tract DCs and lymphocyte effectors to the tumor site could serve
as potent agents in cancer immunotherapy.

CCL21, a CC chemokine expressed in high endothelial
venules and in T-cell zones of the spleen and lymph nodes
strongly attracts naïve T cells and DCs (27–34). Hence, we
postulated that CCL21 delivery to the tumor site might be
beneficial, because it would colocalize T cells and DCs that
may reverse tumor-mediated immune suppression and orchestrate
effective cell-mediated immune responses. The capacity of
CCL21 to attract DCs (35) is a property shared with other
chemokines (36–38). However, CCL21 may be distinctly ad-
vantageous because of its capacity to elicit a type I cytokine
response in vivo (39). In addition to its immunotherapeutic
potential, CCL21 has been found to have potent angiostatic effects (40). We have demonstrated previously that recombinant CCL21 administered intratumorally elicits potent antitumor responses in murine models of established lung cancer (39). Using two transplantable murine lung cancer models, we have shown that the antitumor efficacy of CCL21/SLC is T cell-dependent. The CCL21/SLC-mediated antitumor response was dependent on both CD4 and CD8 lymphocyte subsets, and was accompanied by DC infiltration of the tumor (39). Vicari et al. (41) substantiated the results of our earlier findings in the C26 colon cancer model. Using C26 colon carcinoma cells transduced with the CCL21/SLC cDNA, Vicari et al. (41) demonstrated that the CCL21/SLC-transduced tumor cells had reduced tumorigenicity that was attributed to both immunological and angiostatic mechanisms (41). In recent studies that directly support the angiogenic capacity of this chemokine, Arenberg et al. (42) have reported that CCL21/SLC inhibits human lung cancer growth and angiogenesis in a SCID mouse model.

In this study we evaluated the importance of CCL21 secretion by DCs and the determinants of the antitumor responses in a murine lung cancer model after intratumoral administration of DCs genetically modified to express CCL21/SLC. This approach attempts to use an in situ source of antigen for DCs. In contrast to in vitro immunization with purified peptide antigen, autologous tumor has the capacity to provide the DC administered at the tumor site access to the entire repertoire of available antigens in situ. This may increase the likelihood of a response and reduce the potential for tumor resistance due to phenotypic modulation. We report that intratumoral injection of DC-AdCCL21 is effective in generating systemic antitumor immune responses, and these are dependent on the induction of IFN-γ, MIG/CXCL9, and IP-10/CXCL10.

MATERIALS AND METHODS

Reagents. Antibodies to murine IFN-γ and recombinant IFN-γ and were from PharMingen (San Diego, CA). Antibodies to murine MIG/CXCL9, IP-10/CXCL10, transforming growth factor (TGF) β, and recombinant cytokine standards were purchased from R&D (Minneapolis, MN). Antimurine monoclonal antibody for CCL21 and recombinant CCL21/SLC were purchased from R&D (Rocky Hill, NJ). Biotinylated antimurine antibody for CCL21 was obtained from R&D (Rocky Hill, NJ). Interleukin (IL)-12 determination was performed 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 immunosorbent for IFN-γ was performed using a kit from PharMingen. Polyclonal goat antimurine MIG/CXCL9 and antimurine IP-10/CXCL10-specific antisera were produced and characterized as described previously (43). Antimouse IFN-γ monoclonal (R4–462; American Type Culture Collection, Rockville, MD) neutralizing antibody was purified by affinity chromatography from SCID mice ascites, which was generated 3–4 weeks after i.p. injection of 106 R4–462 hybridoma cells/mouse (44). The dose of each antibody used neutralized >95% of the respective circulating cytokine/chemokine (data not shown). For flow cytometry, labeled antibodies (FITC, phycoerythrin, and peridinin chlorophyll protein) against the T-cell surface markers to CD3, CD4, CD8, and CD25, and against the DC markers, CD11c and DEC 205 and the appropriate controls were purchased from Pharmingen. FITC-labeled antirabbit IgG was purchased from PharMingen. CXCR3 was detected by rabbit antimouse CXCR3 antibody (Zymed Laboratories Inc., South San Francisco, CA) and FITC-conjugated polyclonal antirabbit IgG (PharMingen).

Mice. Pathogen-free Balb/C female mice, 6–8 weeks of age, were obtained from Charles River Laboratories (Hollister, CA) and maintained in the West Los Angeles Veterans Administration Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Research Facility. The institutional animal studies committee approved all of the experiments.

Cell Culture. Murine line 1 alveolar lung tumor (L1C2; Ref. 45) and the WEHI cell line, a 3-methylcholanthrene-induced fibrosarcoma (American type Culture Collection, Manassas, VA) were used in these studies. The cells were routinely cultured as monolayers in 25-cm2 tissue culture flasks containing RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 2 mm glutamine (JRH Biosciences, Lenexa, KS) and maintained at 37°C in humidified atmosphere containing 5% CO2 in air. The cell lines were Mycoplasma free and cells were used up to the tenth passage before thawing frozen stock cells from liquid N2. Fibroblast cultures were established from Balb/C mouse skin. Skin was cut into small pieces, digested in trypsin for 1 h, washed in PBS three times, and plated in a 1:1 mixture of DMEM and Ham’s F11 containing bovine insulin (10 μg/ml), 15 mm HEPES buffer (Life Technologies, Inc., Gaithersburg, MD), human transferrin (25 μg/ml), human high-density lipoprotein (20 μg/ml), human platelet-derived growth factor (1 unit/ml), and mouse epidermal growth factor (100 ng/ml; Sigma, St. Louis, MO). Monolayer cells were trypsinized to obtain single cell suspension for adenosinal transduction.

Tumorigenesis Experiments. L1C2 tumor cells (1.5 × 105) were injected s.c. in the right suprascapular area of Balb/C mice, and tumor volume was monitored three times per week. Five-day-old established tumors were treated with intratumoral injection of 106 DCs administered once a week for 3 weeks. All of the treatments were administered intratumorally once a week for 3 weeks, and treatment groups included DC-AdCCL21, DC, DC-CV, AdCCL21 (106 plaque-forming units; pfu), AdCV, and Fibroblast-AdCCL21. Two biocating diameters of each tumor were measured with calipers. The volume was calculated using the formula V = 0.4ab2 with “a” as the larger diameter and “b” as the smaller diameter. Mice that responded to therapy by completely rejecting their tumors were rechallenged on day 31 with a s.c. injection of 5 × 105 L1C2 cells in the left suprascapular region.

For the evaluation of DC-AdCCL21-mediated systemic antitumor responses, L1C2 cells were inoculated s.c. in the right flank (1.5 × 103 cells) and left flank (2 × 103 cells). All of the treatments were administered intratumorally into the right flank once a week for 3 weeks, and treatments groups included AdCCL21 (104 pfu), Fibroblast-AdCCL21, and DC-AdCCL21.

In Vivo Cytokine Neutralization. To determine the importance of IFN-γ, MIG/CXCL9, and IP-10/CXCL10 in DC-
AdCCL21-mediated antitumor responses, in vivo neutralization experiments were performed. L1C2 tumor cells (1.5 × 10^5) were inoculated by s.c. injection in the right supra-scapular area of Balb/C mice. Five-day established tumors were treated with intratumoral injection of DC-AdCCL21 once a week for 3 weeks. Twenty four h before treatment, and then three times a week, mice were injected i.p. with 1 ml/dose of anti-IP-10/CXCL10 or anti-MIG/CXCL9, or 100 µg/dose of purified monoclonal anti-IFN-γ or appropriate control antibodies (goat IgG and rat IgG) at equivalent doses for the duration of the experiment. Tumor volumes were assessed three times per week. In response to these antibodies, there was a significant reduction of the respective cytokines in vivo (Fig. 6B).

Preparation of Adenoviral Vector Expressing AdCCL21. The adenoviral construct (AdCCL21) is an E1-deleted, replication-deficient adenoviral type 5 vector (Ad5) encoding a 456-bp murine CCL21/SLC cDNA. The CCL21/6Ckine/SLC cDNA was inserted into the former E1 site of the Invitrogen pMH4 plasmid and driven by the cytomegalo-virus promoter-enhancer. The AdCCL21 was prepared through an in vitro recombination event in 293 cells between the shuttle pMH4 plasmid that contained the CCL21/SLC cDNA and pJM17 plasmid, which contains the entire E1, deleted Ad5 genome. The control vector (AdRR5) did not contain the CCL21 cDNA insert. Clones of AdCVC and AdCCL21 were obtained by limiting dilution analysis of the ability of the medium to induce a cytopathic effect on 293 fresh cells and confirmed by CCL21/SLC-specific ELISA. Viral stocks were then obtained by amplification of the 293 cells followed by CsCl purification, dialysis, and storage as a glycerol (10% volume/volume) stock at −80°C. The titer of each viral stock was routinely 10^11−10^13 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.

Isolation and Propagation of Bone Marrow-Derived DCs. DCs 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 obtained from the American Type Culture Collection) and rabbit serum complement (Sigma) for 1 h. Cells were washed and then incubated overnight to allow contaminating macrophages to adhere, and nonadherent DCs were harvested and cultured in vitro for 6 days with murine granulocyte macrophage colony-stimulating factor (GM-CSF; 2 ng/ml) and IL-4 (20 ng/ml; R&D Systems) as described previously (9). Consistent with previous studies from our laboratory as well as others (6, 9, 23, 46), DCs 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% DCs as defined by coexpression of these cell surface antigens (data not shown).

Transduction of DCs and Fibroblasts with Adenoviral 5 Vector. To optimize the multiplicity of infection for CCL21 production, in vitro propagated DCs were transduced on day 7, in RPMI containing 2% fetal bovine serum, for 2 h with AdCCL21 (DCAdCCL21) at multiplicity of infections of 10:1, 20:1, 50:1, and 100:1. CCL21 protein concentrations in transduced DCs or fibroblast supernatants were assessed in vitro from day 1 to day 17. Transduced DCs and fibroblasts produced CCL21 for up to 17 days in culture. Efficiency of transduction of DCs by adenoviral type 5 vector was assessed with an Ad5lacZ vector. DCs (5 × 10^5) were transduced with Ad5lacZ vector (multiplicity of infections of 100:1 and 20:1) in vitro. The DCs were incubated with the virus for 2 h and washed before 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining 24 h after transduction (47). For in vivo experiments, day 7 DCs or fibroblasts were resuspended at a concentration of 1 × 10^7 cells/ml in RPMI 1640 containing 2% fetal bovine serum and transduced for 2 h with AdCCL21 at multiplicity of infection of 100:1. The transduced DCs and fibroblasts produced 7−10 ng/ml/10^6 cells/24 h of CCL21/SLC.

Flow Cytometry. On day 12 after tumor inoculation, flow cytometric analyses for T-cell and DC markers were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) in the University of California Los Angeles Jonsson Cancer Center Flow Cytometry Core Facility. Non-necrotic 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 min 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 lymphocytes from tissue matrix. The cells were pelleted at 2,000 rpm for 10 min and cell pellets washed twice to remove collagenase. Leukocytes were additionally purified using a discontinuous Percoll (Sigma) gradient, collecting at the 35−60% interface after centrifugation at 1,500 rpm for 20 min at 4°C without brake. The collected cells were washed twice in PBS and stained for flow-cytometric evaluation. After Percoll purification, the percentage of leukocytes in the cell population was approximately >95%. Cells were identified as lymphocytes or DCs by gating based on forward and side scatter profiles. CD11c+ DCs were defined as the bright populations within tumor nodules. Gated events (10,000) were collected and analyzed using Cell Quest software (Becton Dickinson). For staining, two or three fluorochromes (phycoerythrin, FITC, and PerCP; PharMingen) were used to gate on the CD3, CD4, and CD8 T-lymphocyte population or CD11c+ DCs in single-cell suspensions from tumor nodule. For CXCR3 expression, T cells were doubly stained for CD3 and CXCR3 cell surface markers. For T-regulatory cell quantification, T cells were doubly stained for CD4 and CD25 cell surface markers.

Cytokine Determination from Tumor Nodules and Spleens by ELISA. The cytokine profiles in tumors and spleens were determined as described previously (9). On day 12 after tumor inoculation, non-necrotic tumors were harvested, cut into small pieces, homogenized, and passed through a sieve (Bellco Glass). Spleens were harvested, teased apart, RBC depleted with double-distilled H_2O, and brought to toxicity with 1× PBS. Tumor nodule homogenates were evaluated for the production of IL-12, GM-CSF, IFN-γ, TGF-β, MIG/CXCL9, and IP-10/CXCL10 by ELISA, and PGE_2 by enzyme immunoassay. Tumor-derived cytokines and PGE_2 concentrations were corrected for total protein by Bradford assay (Sigma), and the results are expressed as pg/mg of total protein. The sensitivities
of the CCL21/SLC, GM-CSF, IFN-γ TGF-β, MIG/CXCL9, and IP-10/CXCL10 ELISAs were 15 pg/ml. For IL-12 the sensitivity was 5 pg/ml. The TGF-β ELISA measured TGF-β1.

**Enzyme-Linked Immunosot.** IFN-γ enzyme-linked immunosorbent assay was performed to determine the frequency of splenocyte-producing IFN-γ in response to irradiated tumors. On day 12 after tumor inoculation, spleens from mice in the various treatment groups were harvested, crushed, RBC depleted, filtered through a 10-μm filter (Millipore, Minneapolis, MN), and coincubated with either irradiated specific L1C2 cell line or the nonspecific syngeneic WEHI cell line at a ratio of 10 lymphocyte effectors:1 stimulator for 24 h. A single cell suspension of L1C2 or WEHI tumor cells (10^6 cells/ml) was irradiated with 80 Gy of gamma irradiation in a 137Cs gamma irradiator. Spots were quantified with an ImmunoSpot Image Analyzer (Cellular Technologies Ltd., Cleveland, OH) at the University of California Los Angeles Immunology Core Facility.

**PGE_2 Enzyme Immunoassay.** PGE_2 concentrations were determined using a kit from Cayman Chemical Co. according to the manufacturer’s instructions as described previously (48). The enzyme immunoassay plates were read by a Molecular Dynamics Microplate Reader (Sunnyvale, CA).

**Statistical Analyses.** Groups of 6–8 mice were used in each experiment. All of the experiments were repeated at least three times. Statistical analyses of the data were performed using the Kruskal-Wallis one-way ANOVA on ranks, followed by multiple pairwise comparisons according to Dunn’s method. Significance at the P < 0.05 level is denoted.

**RESULTS**

**Intratumoral DC-AdCCL21 Generates Systemic Antitumor Responses and Confers Tumor Immunity.** The antitumor efficacy of DC-AdCCL21 was evaluated in Balb/C mice with established L1C2 tumors. Five days after tumor implantation, mice were treated intratumorally with the following treatments weekly for three weeks: (a) Diluent; (b) DCs; (c) DC-AdCCL21; (d) DC-AdCV; (e) fibroblast-AdCCL21; (f) AdCCL21 (10^6 pfu); (g) AdCV; and (h) recombinant CCL21/SLC (10 ng). Tumor growth curve slope analyses were performed to compare the tumor growth rates of the treatments versus the diluent-treated control. After the first two treatments, compared with the diluent-treated controls, the decreases in the tumor growth curve slopes were DC (1.7-fold); DC-AdCCL21 (1.6-fold); DC-AdCV (1.5-fold); fibroblast-AdCCL21 (1.7-fold); and AdCCL21 (2.3-fold). After the third treatment, compared with the diluent-treated controls, the reductions in the tumor growth curve slopes were DC (2.4-fold); DC-AdCCL21 (1.6-fold); DC-AdCV (2.0-fold); fibroblast-AdCCL21 (4-fold); and AdCCL21 (3.2-fold). Intratumoral injection of 10^6 DC-AdCCL21 (7–10 ng/ml/10^6 cells/24 h of CCL21) at weekly intervals for 3 weeks led to a significant reduction in tumor volumes compared with diluent-treated controls, DC, DC-AdCCL21, AdCCL21, and AdCCL21 (Fig. 1, P < 0.01 for DC-AdCCL21 versus the DC or DC-AdCV groups). Although E1-deleted adenoviral vectors have been reported to elicit antiviral CTL responses (49), we found that therapy with DC-AdCV was not more effective than unmodified DCs in reducing tumor burden, as both of these groups mediated a 1.5–2-fold decrease in tumor burden (Fig. 1). Similarly, injection of recombinant CCL21/SLC (10 ng) in the dose range secreted by the transduced DCs did not have antitumor therapeutic efficacy in this model, and the tumor growth curve was indistinguishable from the diluent-treated control. Thus, recombinant CCL21 in the range produced by DC in vitro was ineffective at generating antitumor responses in vivo. In contrast, mice treated with DC-AdCCL21 had a 7-fold reduction in tumor growth rate after the first 2 weeks and a 16-fold reduction after 3 weeks of treatment compared with diluent-treated mice. These findings suggest that CCL21 is a critical element for optimal responses and must be secreted by DCs for effective therapy in this model system (Fig. 1). To determine the importance of DCs as a vehicle for CCL21 delivery, AdCCL21-transduced fibroblasts were administered intratumorally. Although they produced similar levels of CCL21, and were as effective as DC-AdCCL21 after the first two treatments, AdCCL21 transduced fibroblasts were not as effective as DC-AdCCL21 after the third treatment in reducing tumor burden. Five of 8 mice treated with DC-AdCCL21, whereas only 2 of 8 mice treated with fibroblast-AdCCL21 intratumorally, showed complete tumor eradication. Although mice treated with DCs or control vector-modified DCs showed tumor volume reduction when compared with diluent control-treated tumors (P < 0.05),
only 1 of 8 mice treated with unmodified or control vector modified DCs (DC-AdCv) showed complete tumor rejection. Similarly, mice treated with AdCCL21 (10^6 pfu/week × 3) showed tumor growth reduction, but only 1 of 8 mice had complete tumor eradication. Intratumoral injection of 5 × 10^6 or 10^7 pfu AdCCL21 showed antitumor responses that were indistinguishable from the 10^6-pfu AdCCL21 dose. After tumor eradication in mice treated with DC-AdCCL21, tumor immunity was induced as indicated by rejection of a secondary challenge of 5 × 10^3 tumor cells in 5 of 5 mice. To determine the extent of systemic antitumor responses generated as a result of intratumoral injections, Balb/C mice were simultaneously inoculated with 1.5 × 10^3 L1C2 cells in the right flank and with 2 × 10^4 cells in the left flank. Only treatments groups that showed the most significant decreases in tumor volumes in the localized model were used. The following treatment groups were used: diluent control, AdCCL21, fibroblast-AdCCL21, and DC-AdCCL21. Therapeutic injections were administered in the right flank tumors only. In the bilateral tumor model, tumor growth curve slopes compared with the diluent-treated control showed the following reductions in the treatment and contralateral flanks, AdCCL21 (1.5-fold), fibroblast-AdCCL21 (2–3-fold), and DC-AdCCL21 (5–10-fold). Three of 7 mice treated with DC-AdCCL21 showed complete regression of both flank tumors (Fig. 2, A and B). In contrast, none of the mice receiving DC-AdCV had antitumor responses (data not shown). Although injection of fibroblast-AdCCL21 led to systemic reduction in the tumor growth rate, only 1 of 7 mice in this treatment group showed complete tumor eradication only in the treatment flank. Injection of AdCCL21 led to tumor growth reduction on both the flanks; however, none of the treated mice in this group had complete tumor eradication.

Fig. 2 A and B, intratumoral DC-AdCCL21 leads to reduction in growth rates of bilateral tumors. L1C2 tumor cells (1.5 × 10^3) were inoculated on the right suprascapular area and 2 × 10^4 L1C2 tumor cells on the left in BALB/c mice. Five days after tumors were established, mice were treated intratumorally on the right side only with diluent, DC-AdCCL21, AdCCL21 (10^6 plaque-forming units), and fibroblast-AdCCL21. Compared with diluent controls, there was a systemic reduction of the bilateral tumors in the following treatment groups, AdCCL21 and fibroblast-AdCCL21. However, compared with diluent-treated control or fibroblast-AdCCL21 treatment groups, the DC-AdCCL21 group evidenced the most significant systemic reduction in bilateral tumors (P < 0.01, compared with diluent treated control and *, P < 0.05 compared with fibroblast-AdCCL21 treatment group; n = 8 mice/group). Forty three percent of mice treated with DC-AdCCL21 showed complete regression of both flank tumors, whereas only 14 percent of mice treated with fibroblast-AdCCL21 showed complete tumor eradication in the treatment flank only. Results are representative of three independent experiments, bars, ±SD.

**DC-AdCCL21 Enhances the Frequency of DCs and T Cells at the Tumor Site.** Because CCL21 is chemotactic for both T cells and DCs, we hypothesized that intratumoral injections of DC-AdCCL21 would elicit migration of these cell types to the tumor site. Flow-cytometric evaluation of single cell suspensions of tumor nodules showed that compared with diluent treated controls, treatment groups receiving DC, DC-AdCV, AdCCL21, and fibroblast-AdCCL21 had a modest yet significant increase in the frequency of CD4⁺, CD8⁺, and CD3⁺CXCR3⁺ T cells, as well as the DCs expressing CD11c⁺ DEC205⁺ but markedly reduced CD4⁺CD25⁺ T regulatory cells infiltrating the tumors. Intratumoral DC-AdCCL21 produced the most significant increases in the frequency of CD4⁺ (248%), CD8⁺ (71.8%), and CD3⁺CXCR3⁺ T cells (71%), as well as the DCs expressing CD11c⁺ DEC205⁺ (94%) but markedly reduced CD4⁺CD25⁺ T-regulatory cells (41.7%) infiltrating the tumors over controls (Fig. 3).

**DC-AdCCL21 Treatment Promotes Type 1 Cytokine and Antiangiogenic Chemokine Release, as well as a Decline in the Immunosuppressive Mediators TGF-β and PGE₂.** On the basis of previous reports indicating that tumor progression can be modified by host cytokine profiles, we evaluated the cytokine production from tumor sites. The tumor sites were evaluated for the presence of IFN-γ, GM-CSF, IL-12, MIG/CXCL9, IP-10/CXCL10, and TGF-β by ELISA, and PGE₂ by enzyme immunoassay. Compared with diluent-treated controls, treatment groups receiving DC, DC-AdCV, fibroblast-AdCCL21, and AdCCL21 had a modest yet significant increase in type 1 cytokines (IFN-γ and IL-12) and antiangiogenic chemokines (IP-10/CXCL10 and MIG/CXCL9) with a concomitant decrease in the immunosuppressive mediators (PGE₂ and TGF-β) at the tumor sites. In comparison with diluent and the other treatment groups, DC-AdCCL21 produced the most significant increases in type 1 cytokines and antiangiogenic chemokines, and the most substantial decline in the tumor production of immunosuppressive mediators. Compared with tumors...
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Fig. 3 DC-AdCCL21 enhances influx of T-cell subsets and dendritic cells (DC) at the tumor site. Five-day-old established tumors were treated once a week for 2 weeks with diluent, DC, DC-AdCCL21, DC-CV, AdCCL21 (10^6 plaque-forming units), AdCV, and fibroblast-AdCCL21 before tumors were harvested for flow cytometric analysis. Single cell suspensions of non-necrotic tumor nodules were prepared and cell surface staining for T-cell markers CD4 and CD8, and the chemokine receptor CXCR3, as well as the DC marker CD11c and DEC205 were evaluated by flow cytometry. Cells were identified as lymphocytes or DCs by gating on the forward and side scatter profiles; 10,000 gated events were collected and analyzed using Cell Quest software. Compared with the diluent-treated control and the other treatment groups, intratumoral DC-AdCCL21 led to an increase in the frequency of CD4⁺ CD25⁺, CD8⁺, CD3⁺ CXCR3⁺, and CD11c⁺ DEC205⁺ but a decrease in the T-regulatory population CD4⁺ CD25⁺. * P < 0.01 compared with diluent-treated control; † P < 0.05 compared with other treatment groups.

DC-AdCCL21 Therapy Induces Specific T-Cell Responses against L1C2. To evaluate the induction of specificity against the treated tumors, IFN-γ enzyme-linked immunospot assays were performed on splenocytes from: (a) Diluent; (b) DCs; (c) DC-AdCCL21; (d) DC-AdCV; (e) fibroblast-AdCCL21; (f) AdCCL21; or (g) AdCV-treated mice. On day 12 after tumor inoculation, splenocytes were restimulated overnight with irradiated autologous L1C2 or irrelevant syngeneic WEHI control tumors at a ratio of 10:1. Compared with diluent-treated controls, there was a modest yet significantly increased frequency of tumor-specific IFN-γ-producing cells in the treatment groups receiving DCs (2-fold), DC-AdCV (2-fold), AdCCL21 (4-fold), and fibroblast-AdCCL21 (5-fold; P < 0.05). Compared with the other treatment groups, intratumoral DC-AdCCL21 (9-fold) produced the most significant increases in the frequency of tumor-specific IFN-γ-producing cells (P < 0.01). There were minimal responses to the control syngeneic tumor WEHI (Fig. 5).

DC-AdCCL21-Mediated Antitumor Responses Require IFN-γ, MIG/CXCL9, and IP-10/CXCL10. The tumor reductions observed in this model could have been due to participation by T cells secreting IFN-γ leading to inhibition of angiogenesis via MIG/CXCL9 and IP-10/CXCL10, as well as T cell-dependent immunity (50). Hence, we postulated that in addition to IFN-γ, IP-10/CXCL10 and MIG/CXCL9 would also

Fig. 4 A and B, DC-AdCCL21 therapy leads to an induction of Thelper 1 cytokines and a decrease in immunosuppressive molecules. Five-day-old established tumors were treated once a week for 2 weeks with diluent, dendritic cells (DC), DC-AdCCL21, DC-CV, AdCCL21 (10^6 plaque-forming units), AdCV, and fibroblast-AdCCL21, and the tumors were harvested for cytokine analysis. Non-necrotic tumors were cut into small pieces, homogenized, and passed through a sieve. Tumor homogenates were evaluated for the presence of granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ, MIG/CXCL9, IP-10/CXCL10, interleukin (IL)-12, and transforming growth factor (TGF)-β by ELISA and prostaglandin E₂ (PGE₂) by enzyme immunoassay. The cytokine and PGE₂ measurements were normalized to total protein determined in the homogenates by the Bradford kit from Sigma. Results are expressed as pg/mg of total protein. Compared with tumor nodules from the control group, mice treated intratumorally with DC-AdCCL21 had significant increase in GM-CSF, IFN-γ, MIG/CXCL9, IP-10/CXCL10, and IL-12, but a decrease in the immunosuppressive molecules PGE₂ and TGF-β. Bars, ± SE. A. * P < 0.01 compared with diluent treated control; † P < 0.05 compared with other treatment groups. B. *, P < 0.05 compared with diluent-treated controls and other treatment groups; n = 8 mice/group.

from the diluent-treated group, mice treated with DC-AdCCL21 had significant reductions in PGE₂ (2-fold) and TGF-β (4-fold), but an increase in IFN-γ (18-fold), GM-CSF (4.5 fold), IP-10/CXCL10 (4-fold), IL-12 (4-fold), and MIG/CXCL9 (4-fold; Fig. 4, A and B). Similar cytokine patterns were also observed in the spleens of DC-AdCCL21-treated mice (data not shown).
be important contributors to the tumor reduction in the context of DC-AdCCL21 therapy.

To determine the importance of MIG/CXCL9, IP-10/CXCL10, and IFN-γ in the DC-AdCCL21-mediated antitumor response, these cytokines were depleted in DC-AdCCL21-treated mice. Anti-IP-10/CXCL10, MIG/CXCL9, and IFN-γ antibodies each significantly inhibited the antitumor efficacy of DC-AdCCL21 (Fig. 6A; *P < 0.01 compared with the control antibody group). Neutralization of IFN-γ caused a significant decrease in both MIG/CXCL9 and IP-10/CXCL10, indicating that these chemokines are largely IFN-γ dependent. Thus, an increase in IFN-γ at the tumor site in DC-AdCCL21-treated mice could explain the relative increases in IP-10/CXCL10 and MIG/CXCL9. The converse was also observed; IFN-γ production at the tumor site was found to be MIG/CXCL9 and IP-10/CXCL10 dependent as indicated by the fact that neutralization of these cytokines caused a significant decrease in IFN-γ (Fig. 6B). Neutralization of any one of these cytokines caused a concomitant decrease in all three of the cytokines, thus indicating that IFN-γ, MIG/CXCL9, and IP-10/CXCL10 are interdependent in the DC-AdCCL21-mediated antitumor responses.

**DISCUSSION**

Host APCs are critical for the cross-presentation of tumor antigens (1). However, tumors have the capacity to limit APC maturation, function, and infiltration of the tumor site (3, 51–53). In an attempt to stimulate specific antitumor immunity, experimental models and clinical studies are currently evaluating the potent antigen-presenting capacity of DCs combined with single or multiple tumor antigen epitopes (14). However, the problems in using tumor antigen-based immunization strategies include: (a) the potential induction of tolerance (54); (b) the inability to use repeated dosing because of vector-associated neutralization (55); and (c) the limitation of therapy to patients...
whose tumors express defined specific tumor antigens in the context of the correct HLA phenotype (56). We and others have described previously a therapeutic paradigm that overcomes these deficits by intratumoral administration of cytokine gene-modified DCs (26, 57–59). This antitumor DC-based therapy exploits the professional APC as an effective vehicle for cytokine delivery and presentation of multiple tumor antigens in situ. In earlier studies, genetic immunotherapy administered by the intratumoral route led to augmentation of antitumor reactivity (6, 9, 60).

Kirk et al. (25) have shown previously a T cell-dependent antitumor immunity after intratumoral administration of DC-AdCCL21. Using a transplantable murine lung cancer model, we embarked on the current studies to evaluate the determinants of DC-AdCCL21 antitumor therapy as well as the requirement of DCs to secrete CCL21 for effective antitumor immune responses. Our rationale for transducing DCs to express CCL21 was to enhance the antitumor properties of DCs by providing a chemokine at the tumor site that attracts host APCs and T cells. CCL21, a CC chemokine expressed in high endothelial venules, and in T-cell zones of spleen and lymph nodes, strongly attracts naive T cells and DCs (27–34). Because DCs are potent APCs that function as principal activators of T cells, the capacity of CCL21 to facilitate the colocalization of both DCs and T cells may reverse tumor-mediated immunosuppression and orchestrate effective cell-mediated immune responses. In addition to its immunotherapeutic potential, CCL21 has been found to mediate potent angiostatic effects (40) thus adding additional support for its use in cancer therapy. On the basis of these dual capacities we speculated that CCL21 would be an important protein to be expressed from DCs for evaluation in cancer immunotherapy.

The antitumor activity of intratumoral DC-AdCCL21 was determined in a transplantable model for lung cancer. The efficacy of injecting immune stimulators intratumorally for the treatment of cancer has been demonstrated in recent studies; intratumoral injection of recombinant CCL21/SLC evidenced potent antitumor responses in murine lung cancer models (39, 42). In experimental models, DC injection into tumor masses has some antitumor activity against micrometastasis. However, if ex vivo-generated DCs are genetically modified to express IL-12 (58, 61), IL-7 (26), CD40L (62), or IL-2 (63), they are highly efficacious against malignant tumors and elicit specific CTLs. Our rationale for injecting DC-AdCCL21 intratumorally was to colocalize DCs and T cells at the source of tumor antigens where they can prime specific antitumor immune responses. Access to intratumoral injection is achievable in many clinical situations. The inductions of antigen-specific responses require the activation of naive T lymphocytes by APCs bearing cognate antigen. T-cell priming is thought to occur only in specialized compartments (i.e., secondary lymphoid organs such as the spleen and lymph nodes). However, recent work by Kirk et al. (64) has shown that intratumoral administration of SLC gene-modified DCs leads to tumor infiltration by host-derived T cells, which were primed within the tumor mass as determined by expression of activation markers and IFN-γ production. The data suggested that modulation of the tumor microenvironment could lead to effective T-cell priming and the generation of functional antitumor effector cells in the absence of functional lymph nodes.

Our results show that intratumoral injection of DC-AdCCL21 was effective in generating systemic antitumor responses and was accompanied by extensive lymphocyte, as well as DC infiltrates of the tumor sites. The increased CD8+ T-cell infiltrate does not appear to be due to an antiadenovirus response but rather due to CCL21, because treatment groups receiving AdCV or DC-AdCV did not have increased levels of CD8+ T-cell infiltration. The DC-AdCCL21 therapy led to tumor immunity. Unlike DCs, fibroblasts were not a fully effective vehicle for CCL21. We interpret these results as an indication that DCs are necessary for maximal antitumor efficacy. The potent antitumor properties of DC-AdCCL21 demonstrated in this model await additional evaluation in a model in which the tumors arise spontaneously in the lung.

The cytokine production at the tumor site was altered as a result of DC-AdCCL21 therapy. The following cytokines were measured, PGE2, TGF-β, IFN-γ, GM-CSF, IL-12, MIG/CXCL9, and IP-10/CXCL10. These cytokines were evaluated because the tumor site has been documented to be abundant sources of PGE2 and TGF-β that have been shown to suppress immune responses (48, 65) and to promote angiogenesis (66, 67). Antibodies to TGF-β and PGE2 suppress tumor growth in vivo model systems (68, 69). TGF-β is known to suppress antigen presentation, and antagonize CTL generation and macrophage activation (65). DC-AdCCL21-treated tumor-bearing mice showed significant reductions in PGE2 and TGF-β at the tumor sites. Thus, possible benefits of a DC-AdCCL21-mediated decrease in these molecules include promotion of antigen presentation and CTL generation (65), as well as a limitation of angiogenesis (66, 67).

Apart from a decrease in TGF-β and PGE2, the tumor sites of DC-AdCCL21-treated mice revealed significant increases in IFN-γ, IL-12, IP-10/CXCL10, MIG/CXCL9, and GM-CSF. It is well documented that successful immunotherapy shifts tumor-specific T-cell responses to a type 1 cytokine profile (70). Both IL-12 and IFN-γ mediate a range of biological effects that facilitate anticancer immunity. IL-12, a cytokine produced by macrophages (71) and DCs (72), mediates potent antitumor effects that are the result of several actions involving the induction of CTL (73), T-helper 1-mediated immune responses, and natural killer activation (71), as well as the impairment of tumor vascularization (74). An increase in GM-CSF in DC-AdCCL21-treated mice could enhance DC maturation and antigen presentation (75). IP-10/CXCL10 and MIG/CXCL9 are CXC chemokines that chemotactically activate T cells expressing the CXCR3 chemokine receptor (76), and are known to have potent antitumor and antiangiogenic properties (77–80). MIG/CXCL9 and IP-10/CXCL10 are potent antiangiostatic factors that are induced by IFN-γ (50, 79, 81). The tumor reductions observed in this model may be due to T-cell-dependent immunity as well as participation by T cells secreting IFN-γ in inhibiting angiogenesis (50) via induction of MIG/CXCL9 and IP-10/CXCL10. Hence, an increase in IFN-γ at the tumor site in DC-AdCCL21-treated mice could explain the relative increases in IP-10/CXCL10 and MIG/CXCL9. Both MIG/CXCL9 and IP-10/CXCL10 are chemotactic for stimulated CXCR3-expressing T lymphocytes that could further amplify IFN-γ at the tumor site (82). Flow cytometric determi-
nations revealed that both CD4 and CD8 cells, as well as CD3<sup>+</sup>-ve T cells expressing CXCR3, were increased at the in DC-AdCCL21-treated mice.

To determine the importance of MIG/CXCL9, IP-10/ CXCL10, and IFN-γ in the DC-AdCCL21-mediated antitumor response, these cytokines were depleted in DC-AdCCL21-treated mice. Anti-MIG/CXCL9 or IP-10/CXCL10 and anti-IFN-γ each significantly inhibited the antitumor response. Because MIG/CCL9 and IP-10/CXCL10 share the same receptor (CXCR3), one possible explanation of an inhibition in tumor growth is that in vivo neutralization of MIG/CXCL9 and IP-10/ CXCL10 play interrelated roles in the recruitment of CXCR3-activated T cells in DC-AdCCL21-mediated antitumor responses. In vivo depletion of IFN-γ also inhibited the antitumor efficacy of DC-AdCCL21. The fact that neutralization of IFN-γ was efficient at inhibiting DC-AdCCL21-mediated antitumor response may be due to a decrease in the IFN-γ-dependent CXCR3 ligands MIG/CXCL9 and IP-10/CXCL10, indicating that these chemokines are largely IFN-γ dependent. One interesting question is whether IFN-γ, and therefore, presumably MIG/CXCL9 and IP-10/CXCL10, are necessary in the afferent, efferent, or both phases of the immune response. This question will be addressed in future studies. In this model, CXCL9 (MIG) and CXCL10 (IP-10), ligands of the CXCR3 receptor, appear to play redundant roles in the antitumor effect of DC-AdCCL21, because inhibition of a single ligand significantly reduced tumor growth. The increase in type I cytokines may in part be due to an increase in CD8<sup>+</sup> T-cell infiltrates as well as an increase in specificity against autologous tumor; splenocytes from DC- AdCCL21-treated mice had a significantly increased frequency of tumor-specific T cells producing IFN-γ but not to the irrelevant syngeneic control WEHI.

The current study indicates that DC-AdCCL21 administered intratumorally leads to the generation of tumor-specific antitumor responses that are dependent on IFN-γ, MIG/CXCL9, and IP-10/CXCL10. We have reported previously that intratumoral injection of recombinant CCL21 protein mediated antitumor responses that required IFN-γ, MIG/CXCL9, and IP-10/CXCL10 (83). In the previous experiments and current studies the requirement for IFN-γ, MIG/CXCL9, and IP-10/CXCL10 suggests that the CCL21 secreted by DCs constitutes a critical component for the generation of these effector molecules that are responsible for the antitumor response. The potent antitumor properties demonstrated in this model provide a strong rationale for additional evaluation of DC-AdCCL21 regulation of tumor immunity and its use in immunotherapy for lung cancer.

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REFERENCES


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Intratumoral Administration of Dendritic Cells Overexpressing CCL21 Generates Systemic Antitumor Responses and Confers Tumor Immunity

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