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Interleukin-7 Gene-Modified Dendritic Cells Reduce Pulmonary 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 interleukin (IL)-7 (DC-AdIL-7) was evaluated in a murine model of spontaneous bronchoalveolar cell carcinoma. These transgenic mice (CC-10 TAG), expressing the SV40 large T antigen under the Clara cell promoter, develop bilateral multifocal pulmonary adenocarcinomas and die at 4 months as a result of progressive pulmonary tumor burden. Injection of DC-AdIL-7 in the axillary lymph node region (ALNR) weekly for 3 weeks led to a marked reduction in tumor burden with extensive lymphocytic infiltration of the tumors and enhanced survival. The antitumor responses were accompanied by the enhanced elaboration of interferon (IFN)- γ and IL-12 as well as an increase in the antiangiogenic chemokines, IFN- γ -inducible protein 10 (IP-10/CXCL10) and monokine induced by IFN- γ (MIG/CXCL9). In contrast, production of the immunosuppressive mediators IL-10, transforming growth factor (TGF)- β , prostaglandin E₂ (PGE₂), and the proangiogenic modulator vascular endothelial growth factor (VEGF) decreased in response to DC-AdIL-7 treatment. 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 DC-AdIL-7 in regulation of tumor immunity and its use in lung cancer genetic immunotherapy.

OVERVIEW SUMMARY—In our previous studies, intratumoral administration of adenoviral interleukin (IL)-7-transduced dendritic cells (DC-AdIL-7) resulted in specific systemic antitumor immune responses that led to complete tumor regression and long-term immunity. The intratumoral administration of gene-modified dendritic cells (DC) resulted in effective trafficking of transferred DCs to regional lymph nodes. To determine if direct administration of DC-AdIL-7 in the axillary lymph node region (ALNR) would mediate a significant response against pulmonary tumor, we tested the antitumor efficacy of DC-AdIL-7 in a spontaneous murine lung cancer model. In the CC-10 TAG mice, adenocarcinomas develop in an organ-specific manner and, compared to transplantable tumors, the pulmonary tumors in these mice more closely resemble human lung cancer. In this study we demonstrate that ALNR injection of DC-AdIL-7 mediates potent antitumor responses *in vivo* leading to a significant reduction in tumor burden.

INTRODUCTION

THE CENTRAL IMPORTANCE of functional host professional antigen-presenting cells (APCs) in the immune response against cancer has been well defined (Huang *et al.*, 1994). Dendritic cells (DCs) are highly specialized professional APCs with potent capacity to capture, process, and present antigen to T cells (Banchereau and Steinman, 1998). Tumor cells interfere with host DC maturation and function (Gabrilovich *et al.*, 1996; Kobie *et al.*, 2003; Sharma *et al.*, 2003). Thus, to circumvent tumor-mediated inhibition of DC maturation and function *in vivo*, DCs that have undergone cytokine-stimulated maturation *ex vivo* have been utilized in clinical trials and murine cancer models (Zitvogel *et al.*, 1996; Ribas *et al.*, 1997; Sharma *et al.*, 1997; Miller *et al.*, 1998; Nestle *et al.*, 1998; Thurner *et al.*, 1999; Dallal and Lotze, 2000; Banchereau *et al.*, 2001a,b; Schuler *et al.*, 2003;). Antigen-specific cytotoxic T-lymphocyte (CTL) responses have been achieved utilizing *ex vivo* antigen-pulsed DCs (Mayordomo *et al.*, 1995; Boczkowski *et al.*, 1996; Celluzzi *et al.*, 1996; Zitvogel *et al.*, 1996; Nestle *et al.*, 1998; Thurner *et al.*, 1999; Banchereau *et al.*, 2001a; Schuler *et al.*, 2003; Su *et al.*, 2003). In addition, novel tumor antigen delivery systems utilizing cytokine gene-transduced tumor cells and DCs (Sharma *et al.*, 1997; Miller *et al.*, 1998) or fusion of tumor cells with DCs have resulted in induction of antitumor immunity (Gong *et al.*, 1997; Celluzzi and Falo, 1998; Kugler *et al.*, 2000; Parkhurst *et al.*, 2003). Delivery of tumor antigens by *ex vivo* stimulated DCs has been shown to be superior to purified peptides in avoiding CTL tolerization (Toes *et al.*, 1998). Vaccination with multiple tumor antigens may be superior to the use of a single epitope (Toes *et al.*, 1997; Fields *et al.*, 1998) and these responses can be further enhanced by the coadministration of immune-potentiating cytokines (Sharma *et al.*, 1997; Miller *et al.*, 1998; Kirk *et al.*, 2001).

We have demonstrated previously that interleukin (IL)-7-transduced tumor cells administered intratumorally in conjunction with DCs elicit potent antitumor responses in a murine model of established lung cancer (Sharma *et al.*, 1997). IL-7 delivery to the tumor site may be beneficial because it enhances antigen-specific T-cell cytotoxicity (Kos and Mullbacher, 1993) and synergizes with IL-12 in the induction of T-cell proliferation, cytotoxicity, and interferon (IFN)- γ release (Mehrotra *et al.*, 1995). We have shown that IL-7 gene transfer in non-small-cell lung cancer results in modification of tumor phenotype (Sharma *et al.*, 1996). In addition to its direct effects on tumor cells, IL-7 has the capacity to enhance both CTL generation and longevity (Lynch and Miller, 1994; Akashi *et al.*, 1997). IL-7 also downregulates both macrophage and tumor production of transforming growth factor (TGF)- β and thus may serve to limit tumor-induced immune suppression (Dubinett *et al.*, 1993; Miller *et al.*, 1993). TGF- β has been shown to inhibit the antigen-presenting functions and antitumor activity of DC vaccines (Kobie *et al.*, 2003). In addition to its capacity to downregulate TGF- β production, IL-7 can also limit TGF- β signaling (Huang *et al.*, 2002). However, despite these beneficial effects of IL-7, maximal antitumor benefit was demonstrated when bone marrow-derived DCs were administered intratumorally in conjunction with IL-7 gene-modified autologous tumor (Sharma *et al.*, 1997). Based on these studies we performed experiments utilizing transplantable murine lung cancer models in which we demonstrated that intratumoral administration of adenoviral IL-7-transduced dendritic cells (DC-AdIL-7) results in specific systemic antitumor immune responses that lead to complete tumor regression and long-term immunity (Miller *et al.*, 2000). The intratumoral administration of gene-modified DC resulted in effective trafficking of transferred DCs to regional lymph nodes. To determine if direct administration of DC-AdIL-7 in the axillary lymph node region (ALNR) would mediate significant responses against pulmonary tumor, we tested the antitumor efficacy of intranodal injection of DC-AdIL-7 in a spontaneous murine lung cancer model. In the CC-10 TAG mice, adenocarcinomas develop in an organ-specific manner, and in comparison to transplantable tumors, more closely represent the clinical setting encountered in patients with lung cancer.

Here we demonstrate for the first time that ALNR injection of DC-AdIL-7 mediates potent antitumor responses *in vivo* leading to a significant reduction in pulmonary tumor burden.

MATERIALS AND METHODS

CC10 TAg mice

The transgenic CC-10 TAg mice, in which the SV40 large TAg is expressed under control of the murine Clara cell-specific promoter, CC-10, were used in these studies (Magdaleno *et al.*, 1997). All of the mice expressing the transgene developed diffuse bilateral bronchoalveolar carcinoma. Tumor was evident bilaterally by microscopic examination as early as 4 weeks of age. After 3 months of age, the bronchoalveolar pattern of tumor growth coalesced to form multiple bilateral tumor nodules throughout both lungs. 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 VA Greater Los Angeles Healthcare System vivarium and maintained in the animal research facility. All studies were in compliance with the Institutional Review Board. Before each experiment using the CC-10 TAg transgenic mice, presence of the transgene was confirmed by polymerase chain reaction (PCR) of tail biopsies. The 5' primer sequence was SM19-TAG: 5'-TGGACCTTCTAGGTCTTGAAAGG-3', and the 3' primer sequence was SM36-TAG: 5'-AGGCATTCCACCACTGCTCCATT-3'. The size of the resulting PCR fragment is 650 bp and the amplification products were visualized against molecular weight standards on a 1.5% agarose gel stained with ethidium bromide. All of the experiments used pathogen-free CC-10 TAg transgenic mice beginning at 4–5 weeks of age.

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 ATCC, Manassas, VA) and rabbit serum complement (Sigma, St. Louis, MO) for 1 hr. 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, Minneapolis, MN) as previously described (Sharma *et al.*, 1997). Consistent with previous studies from our laboratory as well as others (Inaba *et al.*, 1992; Sharma *et al.*, 1997; Fields *et al.*, 1998; Miller *et al.*, 1998) dendritic cells characterized by flow cytometry were found to have high-level expression of B7-1, B7-2, CD11c, major histocompatibility complex (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).

Fibroblast culture

Fibroblast cultures were established from FVB mouse skin. Skin from FVB mice was isolated and cut into small pieces, digested in trypsin for 1 hr, washed in phosphate-buffered saline (PBS) three times, and plated in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F11 containing bovine insulin (10 µg/ml), 15 mM HEPES buffer (GIBCO, Gaithersburg, MD), human transferrin (25 µg/ml), human high-density lipoprotein (20 µg/ml), human platelet-derived growth factor (1 U/ml), and mouse epidermal growth factor (100 ng/ml) (Sigma). Monolayer cells were trypsinized to obtain single cell suspension for adenoviral transduction.

Transduction of dendritic cells and fibroblasts with adenoviral 5 vector

The adenoviral construct (AdIL-7) is an E1-deleted, replication- deficient adenoviral type 5 vector (Ad5) encoding human IL-7 cDNA (Arthur *et al.*, 1997). The control vector (AdRR5)

did not contain the human IL-7 cDNA. The IL-7 cDNA was inserted into the former E1 site and driven by the cytomegalovirus (CMV) promoter–enhancer. To optimize the multiplicity of infection (MOI) for IL-7 production, *in vitro* propagated DCs were transduced on day 6, in RPMI containing 2% fetal bovine serum (FBS), for 2 hr with AdIL-7 (DC-AdIL-7) at MOIs of 10:1, 20:1, 50:1, and 100:1. IL-7 protein concentrations in transduced DC or fibroblast supernatants were determined by IL-7–specific enzyme-linked immunosorbent assay (ELISA) as previously described (Sharma *et al.*, 1996). IL-7 protein concentrations in transduced DC or fibroblast supernatants were assessed *in vitro* from day 1 to day 17. Transduced DCs and fibroblasts produced IL-7 for up to 17 days in culture. Efficiency of transduction of DCs by adenoviral type 5 vector was assessed with an Ad5LacZ vector. DCs (5×10^6) were transduced with Ad5LacZ vector (MOIs of 100:1 and 20:1) *in vitro*. The DCs were incubated with the virus for 2 hr and washed prior to 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) staining 24 hr posttransduction (Arthur *et al.*, 1997). For *in vivo* injection, an MOI of 20:1 was selected on the basis of an efficiency of transduction of 50% and optimal IL-7 protein production posttransduction. At an MOI of 20:1, DC-AdIL-7 secreted 8 ng/ml per 10^6 cells per 24 hr. Similarly, fibroblasts were transduced at an MOI of 20:1, with similar transduction efficiency. AdIL-7–transduced fibroblasts secreted IL-7 at 7.5 ng/ml per 10^6 cells per 24 hr. DCs and fibroblasts were washed with PBS three times prior to injection. DCs and fibroblasts were also transduced with the control vector at an MOI of 20:1. As additional controls, mice received DCs that did not undergo transduction. These cells are referred to as unmodified DCs in the description of the results.

Therapeutic model in CC10 Tag mice

Beginning at 4–5 weeks of age, CC-10 TAG transgenic mice were injected in the ALNR with one of the following treatments once per week for 3 weeks: (1) diluent; (2) DC-AdIL-7; (3) empty control adenoviral vector-transduced DCs (DC-AdCV); (4) unmodified DCs; (5) fibroblasts transduced with AdIL-7 (Fib-AdIL-7); (6) unmodified DCs plus recombinant IL-7 (5 ng); (7) recombinant IL-7 (5 ng) alone; (8) AdIL-7 (10^9 plaque-forming units [pfu]); and (9) AdCV (10^9 pfu). For groups receiving DCs, a total of 1×10^6 DCs in 100 μ l volume was administered per injection. The specific activity of recombinant IL-7 was 5.6×10^7 U/mg protein (Sterling Winthrop, Malvern, PA). The endotoxin level reported by the manufacturer was less than 0.1 ng/ μ g of IL-7. The dose of recombinant IL-7 selected corresponded to the amount of IL-7 secreted after DC transduction. At 4 months, mice were sacrificed, and lungs were isolated for quantification of tumor surface area. Tumor burden was assessed by microscopic examination of hematoxylin and eosin (H&E)-stained sections with a calibrated graticule (a 1-cm² grid subdivided into 100 1-mm² squares). A grid square with tumor occupying more than 50% of its area was scored as positive, and the total number of positive squares was determined as described previously (Sharma *et al.*, 1999). Ten separate fields from four histologic sections of the lungs from eight mice per group were examined under high-power (20 $\times$ objective). Ten mice from each group were not sacrificed so that survival could be assessed.

Cytokine determination from tumor nodules and spleens

The cytokine profiles in tumors, lymph nodes, and spleens were determined in the treatment groups as described previously (Sharma *et al.*, 1999). Non-necrotic tumors were harvested, cut into small pieces, homogenized and passed through a sieve (Bellco, Vineland, NJ). Cytokines and chemokines were determined from lung tissue homogenates and expressed as picograms per milligram of total protein. Total protein in the homogenates was determined with a Bradford kit from Sigma. Spleens and lymph nodes were harvested, teased apart, red blood cell count (RBC)-depleted with ddH₂O, and brought to tonicity with $1 \times$ PBS. After a 24-hr culture period, tumor nodule, splenocyte, and lymph node supernatant production of IL-10, IL-12, GM-CSF, IFN- γ , TGF- β , vascular endothelial growth factor (VEGF), MIG/CXCL9, and IP-10/CXCL10

were determined by ELISA and prostaglandin E₂ (PGE₂) by enzyme immunoassay (EIA) and results are expressed as picograms per milliliter per 10⁶ cells.

Cytokine ELISA

Cytokine protein concentrations from tumor nodules, spleens and lymph nodes were determined by ELISA as described previously (Sharma *et al.*, 1997). The plates were read at 490 nm with a Molecular Devices Microplate Reader (Sunnyvale, CA). The recombinant cytokines used as standards in the assay were obtained from PharMingen (San Diego, CA). IL-12 (Biosource, Camarillo, CA) and VEGF (Oncogene Research Products, Cambridge, MA) quantities were determined using kits according to the manufacturer's instructions. MIG/CXCL9 and IP-10/CXCL10 were quantified using a modification of a double-ligand method as described previously (Standiford *et al.*, 1990). The MIG/CXCL9 and IP-10/CXCL10 antibodies and protein were obtained from R&D. For the TGF- β ELISA measurements, samples were acidified and hence the active form of TGF- β was measured. The sensitivities of the IL-10, GM-CSF, IFN- γ , TGF- β , MIG/CXCL9, and IP-10/CXCL10 ELISA were 15 pg/ml. For IL-12 and VEGF the ELISA sensitivities were 5 pg/ml.

PGE₂ EIA

PGE₂ concentrations were determined using a kit from Cayman Chemical Co. (Ann Arbor, MI) according to the manufacturer's instructions as described previously (Huang *et al.*, 1998). The EIA plates were read by a Molecular Devices Microplate reader.

Statistical analysis

Groups of 6–10 mice were used. All experiments were repeated at least twice, and the DC-AdIL-7 group was repeated three times. Statistical analyses of the data was performed using the Kruskal-Wallis one-way analysis of variance on ranks, followed by multiple pairwise comparisons according to Dunn's method. Significance at the $p < 0.05$ level is denoted.

RESULTS

DC-AdIL-7 mediates potent antitumor responses in a murine model of spontaneous bronchoalveolar carcinoma

We evaluated the antitumor efficacy of DC-AdIL-7 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 (Magdaleno *et al.*, 1997). Mice expressing the transgene develop diffuse bilateral bronchoalveolar carcinoma and have an average life span of 4 months. Beginning at 4–5 weeks of age, CC-10 TAg transgenic mice were injected in the ALNR with the following treatments weekly for 3 weeks: (1) diluent; (2) DC-AdIL-7; (3) DC-AdCV; (4) unmodified DCs; (5) fibroblasts transduced with AdIL-7 (Fib-AdIL-7); (6) unmodified DCs plus recombinant IL-7 (5 ng); (7) recombinant IL-7 (5 ng); (8) AdIL-7 (10⁹ pfu); and (9) AdCV (10⁹ pfu). At 4 months when the control mice started to succumb because of progressive lung tumor growth, mice were sacrificed in all of the treatment groups, and lungs were isolated and paraffin embedded. H&E staining of paraffin-embedded lung tumor sections from control-treated mice revealed large tumor masses throughout both lungs with minimal lymphocytic infiltration (Fig. 1A and 1B). Compared to diluent-treated controls there was a decrease in the tumor burden in the following treatment groups: DC (1.6-fold), DC-AdCV (1.5-fold); Fib-AdIL-7 (1.9-fold); unmodified DCs plus recombinant IL-7 (1.9-fold); recombinant IL-7 (1.7-fold); AdIL-7 (1.4-fold), and DC-AdIL-7 (10-fold) (Fig. 1C). Although E1-deleted adenoviral vectors have been reported to elicit antiviral CTL responses (Yang *et al.*, 1994), we found that therapy with DC-AdCV was not more effective than unmodified DCs in reducing tumor burden in the CC-10 TAg mice because both of these groups had a 1.5-fold decrease in

tumor burden (Fig. 1A and 1C). Similarly, injection of recombinant IL-7, DC plus recombinant IL-7 or AdIL-7 in CC-10 TAg did not improve the antitumor therapeutic efficacy in this model. In contrast, mice treated with DC-AdIL-7 had a greater than 10-fold ($p < 0.001$) reduction in pulmonary tumor burden compared to diluent-treated mice. These findings suggest that IL-7 is a critical element for optimal responses and must be secreted by DC for effective therapy in this model system (Fig. 1A and 1C). To determine the importance of DCs as a vehicle for IL-7 delivery, AdIL-7-transduced fibroblasts were administered in the ALNR. Although they produced similar levels of IL-7, AdIL-7-transduced fibroblasts were not as effective as DC-AdIL-7 in reducing tumor burden in the CC-10 TAg mice (Fig. 1A and 1C). Whereas diluent-treated control mice revealed large tumor masses throughout both lungs with minimal lymphocytic infiltration, DC-AdIL-7-treated mice had significantly smaller tumor nodules with extensive lymphocyte infiltration. DC-AdIL-7 treatment prolonged survival; median survival was 18 ± 2 weeks for control-treated mice, whereas mice treated with DC-AdIL-7 had a median survival of more than 40 ± 2 weeks ($p < 0.001$). All other therapies resulted in a median survival of 26 ± 2 weeks (Fig. 1D).

DC-AdIL-7 therapy promotes type 1 cytokine and antiangiogenic chemokine release and a decline in the immunosuppressive cytokines TGF- β and VEGF in CC-10 TAg mice

Based on previous reports indicating that tumor progression can be modified by host cytokine profiles (Alleva *et al.*, 1994; Rohrer and Coggin, 1995), we measured the cytokine production from tumor sites and spleen therapy. Lungs, spleens, and lymph nodes were evaluated for the presence of VEGF, IL-10, IFN- γ , GM-CSF, IL-12, MIG/CXCL9, IP-10/CXCL10, and TGF- β by ELISA and for PGE₂ by EIA. Compared to diluent-treated controls treatment groups receiving DC, DC-AdCV, DC plus rIL-7, and AdIL-7 had a modest yet significant increase in type 1 cytokines (IFN- γ , IL-12) and antiangiogenic chemokines (IP-10/CXCL10, MIG/CXCL9) with a concomitant decrease in the immunosuppressive mediators (PGE₂, VEGF, TGF- β) at the tumor sites. In comparison to diluent and the other treatment groups, DC-AdIL-7 produced the most significant increases in type 1 cytokines and antiangiogenic chemokines and the most substantial decline in the pulmonary production of immunosuppressive mediators. Compared to lungs from the diluent-treated group, CC-10 TAg mice treated with DC-AdIL-7 had significant reductions in VEGF (3-fold, $p < 0.05$), TGF- β (2-fold, $p < 0.05$), IL-10 (6-fold, $p < 0.01$) and PGE₂ (32-fold, $p < 0.001$) but an increase in IFN- γ (15-fold, $p < 0.001$), IP-10/CXCL10 (2-fold, $p < 0.05$), IL-12 (3-fold, $p < 0.05$), and MIG/CXCL9 (3-fold, $p < 0.05$) (Fig. 2A, 2B, and 2C). Similar cytokine patterns were also observed in the spleens of DC-AdIL-7-treated mice. Compared to the diluent-treated group, splenocytes from DC-AdIL-7-treated CC-10 TAg mice revealed reduced levels of PGE₂ (5-fold, $p < 0.05$), IL-10 (2-fold, $p < 0.05$), and VEGF (3-fold, $p < 0.05$) but an increase in GM-CSF (23-fold, $p < 0.001$), IFN- γ (8-fold, $p < 0.01$), IL-12 (3-fold, $p < 0.05$), MIG/CXCL9 (4-fold, $p < 0.05$), and IP-10/CXCL10 (4-fold, $p < 0.05$) (Fig. 3A, 3B, and 3C).

DISCUSSION

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 (Schuler *et al.*, 2003). However, the problems in utilizing tumor antigen-based immunization strategies include: (1) the potential induction of tolerance (Dhodapkar and Steinman, 2002); (2) the inability to utilize repeated dosing because of vector-associated neutralization (Rahman *et al.*, 2001); and (3) the limitation of therapy to patients whose tumors express defined specific tumor antigens in the context of the correct HLA phenotype (Dubinett *et al.*, 2000).

We and others have previously described a therapeutic paradigm that overcomes these deficits by intratumoral administration of cytokine gene-modified DCs (Melero *et al.*, 1999; Kirk and Mule, 2000; Miller *et al.*, 2000; Shimizu *et al.*, 2001). 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 (Sharma *et al.*, 1997; Miller *et al.*, 1998; Gambotto *et al.*, 1999). The DC-based intratumoral delivery of the immunopotentiating cytokine IL-7 resulted in potent systemic, specific antitumor immune responses that resulted in eradication of established murine lung cancers and heightened immunogenicity (Miller *et al.*, 2000).

In the models reported previously, the antitumor efficacy of DC-AdIL-7 was determined using transplantable murine or human tumors propagated at subcutaneous sites. We embarked on the current studies to determine the antitumor properties of DC-AdIL-7 in a clinically relevant model of lung cancer in which adenocarcinomas develop in an organ-specific manner. Transgenic mice expressing SV40 large TAg transgene under the control of the murine Clara cell-specific promoter, CC-10, develop diffuse bilateral bronchoalveolar carcinoma, and have an average life span of 4 months (Magdaleno *et al.*, 1997).

In previous studies, intratumoral injection of DC-AdGFP demonstrated the capacity of DCs to migrate to regional and contralateral lymph nodes as well as the spleen (Miller *et al.*, 2000). Trafficking of the gene-modified DCs from the tumor to lymph node sites brings both potent antigen presentation and IL-7 in proximity to primary immune effector cells, thus augmenting systemic antitumor reactivity. Based on trafficking of gene-modified DCs to the regional lymph nodes after intratumoral administration observed in our previous studies, we hypothesized that injection of DC-AdIL-7 in the ALNR would lead to antitumor responses. The antitumor activity of DC-AdIL-7 was determined in the spontaneous model for lung cancer by injecting DC-AdIL-7 into the ALNR of the transgenic mice. Our rationale for injecting DC-AdIL-7 in the lymph node region was to provide for nodal delivery of IL-7 at the immediate cellular interface of the intimate interactions between DCs and lymphocyte effectors where they can prime specific antitumor immune responses. The efficacy of lymph node injection with immune stimulators for the treatment of cancer has been demonstrated in recent studies; vaccination with tumor cell-DC hybrids in the lymph node region led to regression of human metastatic renal cell carcinoma (Kugler *et al.*, 2000). In many clinical situations access to lymph node sites for injection may also be more readily achievable than intratumoral administration. The ALNR was chosen as a site for injection because of ease of repetitive administration, rather than any documented drainage pattern of tumor cells from the lung. It is quite probable that any secondary antigen-presenting site may be an effective route for induction of therapeutic antitumor responses; however, this hypothesis needs to be tested in future experiments. Our results show that this approach is effective in generating systemic antitumor responses. DC-AdIL-7 injected in the axillary lymph node regions of the CC-10 TAg mice evidenced potent antitumor responses with reduced tumor burden and a survival benefit compared to CC-10 TAg mice receiving diluent control injections. Unlike DCs, fibroblasts were not effective vehicles for IL-7. We interpret these results as an indication that DCs are necessary for maximal antitumor efficacy.

We previously found specific enhanced splenocyte release of IFN- γ and GM-CSF after treatment with DC-AdIL-7 (Miller *et al.*, 2000). The specific cytokine release and intracellular cytokine analysis data from *in vitro* studies were consistent with the role of the DC-AdIL-7 in antitumor responses *in vivo* (Miller *et al.*, 2000). Consistent with these findings, the cytokine production from tumor sites and spleens in the CC-10 TAg mice was altered as a result of DC-AdIL-7 therapy. The following cytokines were measured: VEGF, IL-10, PGE₂, TGF- β , IFN- γ , GM-CSF, IL-12, MIG/CXCL9, and IP-10/CXCL10 (Figs. 2 and 3). The production of these

cytokines was evaluated for the following reasons: the tumor site has been documented to be an abundant source of PGE₂, VEGF, IL-10, and TGF- β , and these mediators have been shown to suppress immune responses (Gabrilovich *et al.*, 1996; Huang *et al.*, 1998; Bellone *et al.*, 1999). VEGF, PGE₂, and TGF- β promote angiogenesis (Ferrara, 1995; Fajardo *et al.*, 1996; Tsujii *et al.*, 1998). Antibodies to VEGF, TGF- β , PGE₂, and IL-10 have the capacity to suppress tumor growth in *in vivo* model systems. VEGF has also been shown to interfere with DC maturation (Gabrilovich *et al.*, 1996). Both IL-10 and TGF- β are immune inhibitory cytokines that may potently suppress antigen presentation and antagonize CTL generation and macrophage activation (Bellone *et al.*, 1999; Sharma *et al.*, 1999). Although at higher pharmacologic concentrations IL-10 may cause tumor reduction, physiologic concentrations of this cytokine suppress antitumor responses (Halak *et al.*, 1999; Sharma *et al.*, 1999; Sun *et al.*, 1999; Stolina *et al.*, 2000; Yang and Lattime, 2003). Prior to DC-AdIL-7 treatment in the transgenic tumor-bearing mice, the levels of the immunosuppressive proteins VEGF, PGE₂, and TGF- β were elevated compared to the levels in normal control mice (data not shown). There was no such increase in IL-10 levels. Similarly there were not significant alterations in IL-4 and IL-5 accompanying DC-AdIL-7 therapy (data not shown). DC-AdIL-7-treated CC-10 TAg mice showed significant reductions in VEGF, TGF- β , IL-10, and PGE₂. The decrease in immunosuppressive cytokines was not limited to the lung but was also evident systemically. Thus, possible benefits of a DC-AdIL-7-mediated decrease in these cytokines include promotion of antigen presentation and CTL generation (Bellone *et al.*, 1999; Sharma *et al.*, 1999), as well as a limitation of angiogenesis (Ferrara, 1995; Fajardo *et al.*, 1996; Tsujii *et al.*, 1998). In future experiments, we will address the significance of the decrease in immunosuppressive factors in response to DC-AdIL-7 therapy.

Successful immunotherapy shifts tumor-specific T-cell responses from a type 2 to a type 1 cytokine profile (Hu *et al.*, 1998). Responses depend on IL-12 and IFN- γ to mediate a range of biologic effects, which facilitate anticancer immunity. IL-12, produced by macrophages (Trinchieri, 1998) and DC (Johnson and Sayles, 1997), plays a key role in the induction of cellular immune responses (Ma *et al.*, 1997). IL-12 mediates potent antitumor effects that are the result of several actions involving the induction of CTL, type 1-mediated immune responses, and natural killer activation (Trinchieri, 1998), as well as the impairment of tumor vascularization (Voest *et al.*, 1995). IP-10/CXCL10, and MIG/CXCL9 are CXC chemokines that chemoattract activated T cells expressing the CXCR3 chemokine receptor (Loetscher *et al.*, 1996). Both IP-10/CXCL10 and MIG/CXCL9 are known to have potent antitumor and antiangiogenic properties (Brunda *et al.*, 1993; Luster and Leder, 1993; Arenberg *et al.*, 1996; Sgadari *et al.*, 1997). The lungs of DC-AdIL-7-treated CC-10 TAg mice revealed significant increases in IFN- γ , IL-12, IP-10/CXCL10, MIG/CXCL9, and GM-CSF. MIG/CXCL9 and IP-10/CXCL10 are potent angiostatic factors that are induced by IFN- γ (Strieter *et al.*, 1995; Arenberg *et al.*, 1996; Tannenbaum *et al.*, 1998) and may be responsible in part for the tumor reduction in CC-10 TAg mice following DC-AdIL-7 administration. Because MIG/CXCL9 and IP-10/CXCL10 have antiangiogenic effects (Brunda *et al.*, 1993; Luster and Leder, 1993; Arenberg *et al.*, 1996; Sgadari *et al.*, 1997), the tumor reductions observed in this model may be attributable to T-cell-dependent immunity as well as participation by T cells secreting IFN- γ and inhibiting angiogenesis (Tannenbaum *et al.*, 1998). Hence, an increase in IFN- γ at the tumor site of DC-AdIL-7-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 additionally amplify IFN- γ at the tumor site (Farber, 1997). An increase in GM-CSF in DC-AdIL-7-treated mice could enhance DC maturation and antigen presentation (Banchereau and Steinman, 1998). We postulate that the antitumor responses seen in this model are T-cell-dependent. However, additional studies are necessary to precisely define the T-cell subsets and the host cytokines that are critical to the DC-AdIL-7-mediated antitumor response.

Taken together, the current study indicates that DC-AdIL-7 injected in the ALNR in this spontaneous lung cancer model leads to the generation of systemic antitumor responses. Host APC are critical for the cross-presentation of tumor antigens (Huang *et al.*, 1994; Banchereau and Steinman, 1998). However, tumors have the capacity to limit APC maturation, function, and infiltration of the tumor site (Gabrilovich *et al.*, 1996; Kobie *et al.*, 2003; Sharma *et al.*, 2003). The current model system overcomes this detriment by activating DC precursors *ex vivo* in GM-CSF and IL-4. This allows DC propagation to occur in an environment conducive to full activation without interference from deleterious tumor-derived products. Although DCs have been implicated in promoting tolerance in chronic inflammatory states (Kronin *et al.*, 1996), tolerance induction may occur when DCs are in a low state of activation or maturation, resulting in decreased lymph node recruitment of mature DCs (Sallusto *et al.*, 1995) or the generation of T-regulatory cells (Roncarolo *et al.*, 2001; Dhodapkar and Steinman, 2002). Thus the use of activated DC in the ALNR may help recruit host DC that are already primed with tumor antigens to these sites and help initiate and/or maintain antitumor immune responses. Hence, the antitumor properties of DC-AdIL-7 may be attributable to the stimulation of host antigen-presenting functions by IL-12 as well as antiangiogenic activities mediated via IFN- γ induction of IP-10/CXCL10 and MIG/CXCL9. Secretion of IL-7 by DCs at the nodal sites may also increase the longevity of the activated T cells (Lynch and Miller, 1994; Akashi *et al.*, 1997). Additional studies will be required to delineate the importance of each of these cytokines in DC-AdIL-7-mediated antitumor responses and the precise role of the DCs in this model await definition in further studies. The potent antitumor properties demonstrated in this model of spontaneous bronchoalveolar carcinoma provide a strong rationale for additional evaluation of DC-AdIL-7 in the regulation of tumor immunity.

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FIG. 1.

A, B, and C: DC-AdIL-7 mediates potent antitumor responses in a murine model of spontaneous lung cancer. Beginning at 4–5 weeks of age, CC-10 TAg transgenic mice were injected in the axillary lymph node region (ALNR) with the following treatments once per week for 3 weeks. Treatment groups included: (1) diluent; (2) DC-AdIL-7; (3) empty control adenoviral vector-transduced DCs (DC-AdCV); (4) unmodified dendritic cells (DCs); (5) fibroblasts transduced with AdIL-7 (Fib-AdIL-7); (6) unmodified DCs plus recombinant interleukin (IL)-7 (5 ng); (7) recombinant IL-7 (5 ng); (8) AdIL-7 (10^9 plaque-forming units [pfu]); and (9) AdCV (10^9 pfu). **A:** Hematoxylin and eosin (H&E) staining of paraffin-embedded lung tumor sections from control-treated mice evidenced large tumor masses

throughout both lungs without detectable lymphocytic infiltration. Compared to diluent-treated controls there was a decrease in the tumor burden in the following treatment groups: DC, DC-AdCV; Fib-AdIL-7; unmodified DCs plus recombinant IL-7; recombinant IL-7; AdIL-7. However, compared to diluent control group and other therapies, the DC-AdIL-7 group evidenced extensive lymphocytic infiltration with marked reduction in tumor burden (Magnifications, 1×, 40×, and 100×). **B:** Arrows depict tumor (*1) and infiltrate (*2). (Magnifications, 400×). **C:** Tumor burden was quantified within the lung by microscopy of H&E-stained paraffin-embedded sections. Although all treatment groups showed a decrease in tumor burden, DC-AdIL-7 treatment led to the greatest reduction ($p < 0.001$) compared to diluent-treated control (C) ($n = 8$ mice/group). **D:** Survival was prolonged in the DC-AdIL-7 treatment group ($p < 0.05$ compared to the various treatment groups and $p < 0.001$ as compared to diluent-treated control).

FIG. 2.

A–C: DC-AdIL-7 therapy promotes type 1 cytokine and antiangiogenic chemokine release and a decline in the immunosuppressive cytokines transforming growth factor (TGF)- β and vascular endothelial growth factor (VEGF) in the lungs of CC-10 TAg mice. After axillary lymph node region (ALNR) injection of (1) diluent; (2) DC-AdIL-7; (3) empty control adenoviral vector-transduced DCs (DC-AdCV); (4) unmodified dendritic cells (DCs); (5) fibroblasts transduced with AdIL-7 (Fib-AdIL-7); (6) unmodified DCs plus recombinant interleukin (IL)-7 (5 ng); (7) recombinant IL-7 (5 ng); (8) AdIL-7 (10^9 plaque forming units [pfu]); and (9) AdCV (10^9 pfu), pulmonary cytokine profiles in CC-10 TAg mice were determined and compared to those in diluent-treated tumor-bearing control mice. Cytokine and prostaglandin E₂ (PGE₂)

from the lung tissue are expressed as picograms per milligram of total protein. Compared to lungs from diluent-treated CC-10 tumor-bearing mice, CC-10 mice treated with DC-AdIL-7 had significant reductions in IL-10, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)- β , and PGE₂ but a significant increase in interferon (IFN)- γ , IL-12, IP-10/CXCL10 and MIG/CXCL9 (A, B, and C). Values given reflect mean $\pm$ standard error (SE) for six mice per group. * $p < 0.05$ compared to diluent-treated control; $\blacklozenge p < 0.05$ compared to the DC-AdIL-7 treatment group.

FIG. 3.

A–C: DC-AdIL-7 therapy promotes type 1 cytokine and antiangiogenic chemokine release systemically in CC-10 TAG mice. After axillary lymph node region (ALNR) injection of: (1) diluent; (2) DC-AdIL-7; (3) empty control adenoviral vector-transduced DCs (DC-AdCV); (4) unmodified DCs; (5) Fibroblasts transduced with AdIL-7 (Fib-AdIL-7); (6) unmodified dendritic cells (DCs) plus recombinant interleukin (IL)-7 (5 ng); (7) recombinant IL-7 (5 ng); (8) AdIL-7 (10^9 plaque-forming units [pfu]); and (9) AdCV (10^9 pfu), spleen cytokine profiles in CC-10 TAG mice were determined and compared to those in diluent-treated tumor bearing control mice. Cytokine and prostaglandin E₂ (PGE₂) from the spleen are expressed as picograms per milliliter per 10^6 cells. Compared to diluent-treated CC-10 TAG mice,

splenocytes from DC-AdIL-7-treated CC-10 mice had reduced levels of PGE₂ and vascular endothelial growth factor (VEGF) but significant increases in granulocyte macrophage-colony stimulating factor (GM-CSF), interferon (IFN)- γ , IL-12, MIG/CXCL9, and IP-10/CXCL10 (A, B, and C). Values given reflect mean $\pm$ standard error (SE) for six mice per group. * $p < 0.05$ compared to diluent-treated control; $\blacklozenge p < 0.05$ compared to the DC-AdIL-7 treatment group.