

Phase I Study of Liposome–DNA Complexes Encoding the Interleukin-2 Gene in Dogs with Osteosarcoma Lung Metastases

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ABSTRACT

Systemic gene delivery using cationic liposome–DNA complexes (LDCs) has been shown to elicit potent anti-tumor activity in mice with tumor metastases to the lungs. However, intravenous gene delivery for treatment of established cancer has not been evaluated previously in a spontaneous, large animal model. We therefore evaluated the safety, toxicity, and efficacy of intravenous gene delivery, using LDCs in dogs with established tumor metastases. Twenty dogs with chemotherapy-resistant osteosarcoma metastases to the lungs received a series of intravenous infusions of cationic liposomes and plasmid DNA encoding the canine interleukin-2 (IL-2) cDNA. Effects of intravenous gene delivery on immune activation, clinical and hematologic parameters, tumor responses, and survival times were assessed. We found that slow intravenous administration of IL-2 LDCs resulted in detectable IL-2 transgene expression in lung tissues of dogs. Repeated intravenous infusions of LDCs were well tolerated by dogs with lung tumor metastases and elicited systemic immune activation, as reflected by fever, leukogram changes, monocyte activation, and increased natural killer cell activity. Three of 20 dogs experienced partial or complete regression of lung metastases after infusion of IL-2 LDCs. Overall survival times were significantly increased in treated dogs compared with historical control animals with the same stage of disease. We conclude that repeated intravenous infusion of LDCs in cancer-bearing dogs is safe and well tolerated at low doses and may be capable of eliciting antitumor activity in some animals with advanced tumor metastases.

OVERVIEW SUMMARY

The safety and toxicity of intravenous gene delivery, using liposome–DNA complexes, were evaluated in a phase I study in 20 pet dogs with osteosarcoma metastases to the lungs. Dogs were treated with twice-monthly intravenous infusions of LDCs containing the canine interleukin-2 cDNA and the effects on immune function, clinical and hematologic parameters, and overall survival times were assessed. Slow intravenous infusions of LDCs elicited strong immune activation in dogs, accompanied by fever, leukopenia, and increased natural killer cell activity. Repeated infusions of low doses of LDCs were well tolerated clinically. Three of 20 dogs experienced clinical responses in lung tumor nod-

ules. Overall survival times were significantly increased in treated dogs compared with historical control dogs with osteosarcoma matched for age and stage of disease. We conclude that these results in a large animal spontaneous tumor model validate the potential effectiveness of intravenous gene delivery using LDCs as a new approach to management of lung tumor metastases in humans.

INTRODUCTION

INTRAVENOUS GENE DELIVERY based on liposome–DNA complexes (LDCs) has been shown by several groups to elicit substantial antitumor activity (Dow *et al.*, 1999a; Whitmore *et*

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et al., 1999, 2001; Bramson *et al.*, 2000; Higgins *et al.*, 2004). Genes evaluated by this approach have included cytokine genes (e.g., interleukin [IL]-2, IL-12, and interferon [IFN]- γ), angiogenesis-inhibiting genes (e.g., endostatin), and apoptosis-inducing genes (Dow *et al.*, 1999a; Liu *et al.*, 1999; Natsume *et al.*, 2000; Li and Ma, 2001; Bianco *et al.*, 2003; Sakurai *et al.*, 2003; Li *et al.*, 2004). The majority of transgene expression after intravenous administration of LDCs was shown to occur in the lungs, which makes this route of delivery particularly desirable for targeting tumors growing in the lungs (Liu *et al.*, 1997, 1999; Templeton *et al.*, 1997; Thierry *et al.*, 1997; Anwer *et al.*, 2000). For example, we showed previously that cytokine gene delivery targeted to the lungs by intravenous LDCs could significantly reduce the lung tumor burden in several different tumor models (Dow *et al.*, 1999a). Several key questions regarding the translation of systemic nonviral gene delivery to humans and other larger species have, however, not yet been addressed. For example, what doses of LDCs would be required to produce gene expression *in vivo* in larger animals? Would the administration of complexes need to be modified, because the bolus injections typically done in mice would not be feasible or safe? Could therapeutic activity be elicited at doses that would not elicit serious toxicity, because LDCs are known to also trigger potent activation of innate immunity and inflammatory responses (Dow *et al.*, 1999b; Whitmore *et al.*, 1999; Bramson *et al.*, 2000; Siders *et al.*, 2002)?

To help address these questions, we conducted studies of intravenous LDC-mediated gene therapy in normal rabbits and purpose-bred dogs, as well as in pet dogs with spontaneous lung tumor metastases. Dogs share many physiologic features with humans, including similar body size and similar metabolism and drug distribution kinetics, and dogs have been shown to be much more closely related genetically to humans than are rodents (Vail and MacEwen, 2000; Kirkness *et al.*, 2003). Moreover, dogs develop spontaneous tumors at a rate similar to humans and are a relatively outbred species from an immunological standpoint. For these reasons, preclinical studies of novel anticancer agents in dogs with cancer can yield important insight into expected outcomes in eventual human studies. Osteosarcoma in dogs is a common and highly metastatic tumor that biologically closely resembles osteosarcoma in humans (Misdorp, 1980; Pelfrene, 1985; MacEwen, 1990; Vail and MacEwen, 2000). Moreover, dogs with metastatic osteosarcoma do not have other treatment options once adjuvant chemotherapy has failed.

Twenty dogs with chemotherapy-resistant lung metastases from osteosarcoma were treated with repeated intravenous infusions of LDCs encoding the canine IL-2 gene. The purposes of this study were to determine a safe and tolerated dose of LDCs for intravenous gene therapy, to assess gene expression, to assess immunologic responses to intravenous gene delivery, and to assess the effects of treatment on tumor responses in the lungs and survival times. We found that the maximum tolerated dose of LDCs in cancer-bearing dogs was approximately 20 $\mu\text{g}/\text{kg}$ body weight when administered as a slow infusion over 90 min. At this dose, most dogs developed transient fever responses and significant leukogram changes that largely resolved within 24 hr of infusion. IL-2 gene expression was detected in lung tissues but not in other tissues of treated dogs. Repeated administrations were well tolerated and

did not produce cumulative toxicity. Intravenous infusion of LDCs resulted in rapid systemic immune activation that subsided over the next 24 to 48 hr. Objective tumor responses occurred in 3 of the 20 treated dogs, along with significant prolongation in overall survival times compared with historical control animal data. From these studies it was concluded that intravenous cytokine gene delivery could be successfully scaled up to larger animals and that this treatment could elicit substantial antitumor activity, even in animals with advanced tumor metastases.

MATERIALS AND METHODS

Preparation of expression vectors and plasmid DNA

The cDNA for canine IL-2 was cloned from normal dog peripheral blood mononuclear cells (PBMCs) and expression was confirmed *in vitro*, as reported previously (Dow *et al.*, 1998). In addition, the cDNA for human IL-2 was synthesized (Operon Biotechnologies, Huntsville, AL). Both constructs were placed in a plasmid-based eukaryotic expression vector (pMB75.6) that utilized the cytomegalovirus (CMV) immediate-early promoter-enhancer region, a synthetic intron (pGL3) immediately upstream of the start site, a simian virus 40 (SV40) early poly(A) site, and the kanamycin resistance gene, as described previously (Fairman *et al.*, 1999). The pMB75.6 plasmid without an insert ("empty vector") was also used in some infusions. Plasmid DNA was propagated in *Escherichia coli* and isolated by alkaline lysis, followed by column chromatography, as reported previously (Liu *et al.*, 1997). The endotoxin content of the DNA as determined by *Limulus* amoebocyte lysate (LAL) assay (Cambrex Bio Science, Walkersville, MD) was less than 0.25 EU/ μg DNA. A stock DNA solution (prepared in a 10 mM solution of Tris-HCl [2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride], pH 8.0, at 5.0 mg/ml) was diluted to 0.625 mg/ml with 5% (w/v) dextrose in water (D5W).

Formulation of liposomes and liposome-DNA complexes

Liposomes were prepared by dissolving cholesterol (Sigma, St. Louis, MO) and the cationic lipid 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolium chloride (DOTIM; Sigma-Aldrich, St. Louis, MO) in chloroform (EM Science, Gibbstown, NJ) at equimolar concentrations in a 3-liter round-bottom flask. The clear solution was rotated on a Buchi Rotavapor R-134 (Böchi Labor Technik, Zurich, Switzerland) at 30°C for 30 min and hydrated in 5% (w/v) dextrose (B. Braun Medical, Irvine, CA) to produce a nominal concentration of 40 mM. The liposome dispersion was extruded through successive filters (0.4, 0.2, 0.1, and 0.05 μm) to obtain a vesicle size of approximately 100 nm as measured with a Nicomp C-370 particle sizer (Particle Sizing Systems, Santa Barbara, CA). Immediately before infusion, LDCs were prepared by first dissolving liposomes in 5% dextrose in water at a concentration of 100 μl of liposomes per 1 ml of 5% dextrose solution. Next, the plasmid DNA was added to the liposome solution at a final concentration of 100 μg of DNA per milliliter of solution and complexes were formed by gentle pipetting. The LDCs were infused within 15 min of preparation.

Intravenous delivery of LDCs

Before treatment, all dogs had an indwelling intravenous catheter placed in either the cephalic vein or the medial saphenous vein, without sedation. Using a syringe pump (Harvard Apparatus/Harvard Bioscience, Holliston, MA), the LDC solution was infused at a continuous rate over a 90-min period. This rate of infusion and concentration of LDCs were selected for optimal *in vivo* transfection efficiency, on the basis of preclinical studies of intravenous LDC infusion in rabbits (D. Liggitt, unpublished data). Thus, for a typical 25-kg dog receiving a 20- $\mu\text{g}/\text{kg}$ dose of plasmid DNA, the total DNA dose delivered was 500 μg , and the infusion rate for the LDC solution was 55 $\mu\text{l}/\text{min}$.

Determination of gene expression in tissues of normal dogs after intravenous infusion of LDCs

Three healthy beagle dogs of approximately 15 kg body weight were infused intravenously with plasmid DNA (20 $\mu\text{g}/\text{kg}$) encoding the human IL-2 cDNA, using LDCs prepared as described above. The protocols for these studies were approved by the Institutional Animal Care and Use Committee at Biosupport (Redmond, WA). A fourth dog served as a control and was infused with liposomes only. Twenty-four hours postinfusion, the dogs were humanely killed, tissues (lung, spleen, kidney, heart, liver, and lymph nodes) were collected, and a portion was snap-frozen for analysis of gene expression and also processed for routine histopathology. For quantitation of human IL-2 concentrations in tissues, the tissues were homogenized in lysis buffer as described previously (Dow *et al.*, 1999a) and then centrifuged to clarify the supernatants. The protein concentration in tissue homogenates was determined by bicinchoninic acid (BCA) assay (Bio-Rad, Hercules, CA). The tissue supernatants from each tissue were then assayed in triplicate at various dilutions for detection of human IL-2 protein, using a commercial enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Controls included lysis buffer alone as well as tissue homogenates from normal dogs and from the dog infused with liposomes only. The concentration of IL-2 was determined by comparison with a standard curve generated with known quantities of recombinant human IL-2. Data were calculated as picograms of human IL-2 per milligram of tissue protein. Tissues were also fixed in formalin and embedded in paraffin, and then sectioned and stained with hematoxylin. An experienced pathologist (D.L.) reviewed the tissue sections for evidence of pathologic changes.

Design of clinical study in dogs with metastatic osteosarcoma

A clinical study of 20 client-owned dogs with metastatic osteosarcoma to the lungs was designed to determine (1) a safe dose for intravenous infusion of LDCs in cancer-bearing dogs, and (2) whether treatment elicited immune activation and antitumor activity. The protocols for this study were approved by Institutional Animal Care and Use Committees at the Veterinary Referral Center of Colorado (Englewood, CO), the National Jewish Medical and Research Center (Denver, CO), and the University of Wisconsin (Madison, WI). Animals eligible for entry into the study had a previous histologic diagnosis of

appendicular osteosarcoma, followed by amputation of the affected limb, followed by conventional adjuvant chemotherapy with doxorubicin or with cisplatin or carboplatin (Vail and MacEwen, 2000). All dogs had subsequently developed radiographically visible lung metastases. Thus, dogs that were entered into this study had failed conventional adjuvant chemotherapy and developed chemotherapy-resistant tumor metastases. Excluded from the study were any dogs with concurrent medical disorders and any dog receiving concurrent immune suppressive therapy such as prednisone.

The treatment schedule was a series of 12 intravenous gene infusions administered once weekly for a total of 12 weeks of treatment. At the end of the 12-week period, a final set of thoracic radiographs was examined for evidence of tumor response. For those dogs with a partial tumor response at 12 weeks (>50% reduction in overall size of the major tumor nodules in the lung), treatment with intravenous IL-2 gene delivery was then continued on a once-monthly basis. A within-patient dose escalation component of the study was utilized for the first five dogs enrolled in the study to determine the maximally tolerated dose of gene delivery. The DNA-dosing increments were based on the total amount of plasmid DNA delivered to each animal. Initial dosing was begun at a starting dose of 5 μg of plasmid DNA per kilogram body weight (this dose was based on preclinical studies in rabbits) and then increased by doubling the dose over subsequent treatments until clinical signs of toxicity developed (vomiting, diarrhea, increased respiratory rate, and/or dyspnea).

Clinical monitoring

Body temperature was determined at 2-hr intervals for the first 24 hr postinfusion for the first treatment. In addition, blood for a complete blood count was collected pretreatment and again at 24 hr posttreatment, and again at week 2, week 8, and week 12. A biochemical panel was performed pretreatment and at weeks 2, 6, and 12 of the study. Tumor responses in the lungs were monitored by serial thoracic radiographs obtained pretreatment and at weeks 4, 8, and 12 of the study. For dogs that received additional treatments beyond the 12-week period, thoracic radiographs were done at 6-month intervals.

Immunologic monitoring

Heparinized whole blood was collected pretreatment, again at 24 hr posttreatment, and again at weeks 2, 4, 8, and 12 of the study. Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll density gradient centrifugation. The PBMCs were immunostained for flow cytometric analysis to assess the effects of treatment on lymphocyte and monocyte subsets and upregulation of activation markers (MHC class II and B7.2). Antibodies against canine CD4, CD8, CD11b, and MHC class II were kindly provided by P. Moore (University of California at Davis, Davis, CA). Antibodies to CD4 and CD8 were conjugated with fluorescein isothiocyanate (FITC), whereas unlabeled antibodies to canine CD11b and MHC class II were used, followed by phycoerythrin (PE)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). A cross-reactive, PE-conjugated antibody to mouse B7.2 (BD Biosciences Pharmingen, San Diego, CA) was also used for detection of costimulatory molecule expression on monocytes. For detec-

tion of IL-2 receptor expression by T cells, biotinylated human IL-2 and PE-conjugated streptavidin (Quantikine from R&D Systems [Minneapolis, MN]; and BD Biosciences Pharmingen, respectively) were used. After blocking nonspecific staining with normal dog serum and human IgG, cells were immunostained and then analyzed with a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Gates for analysis of lymphocyte and monocyte populations were determined by forward and side scatter characteristics, and these populations were analyzed for percentage of CD4⁺ and CD8⁺ T cells and CD11b⁺ monocytes. Data analysis was done with CellQuest software.

Spontaneous natural killer (NK) cell activity was assessed with canine thyroid adenocarcinoma cells (CTACs; kindly provided by S. Helfand, University of Wisconsin) as target cells. PBMCs were collected from dogs at various times during the study and were assayed for cytolytic activity without prior culture or activation. The PBMCs were added to duplicate wells of V-bottom Linbro plates in 100 μ l of medium and then serially diluted to achieve effector-to-target ratios between 400:1 and 25:1. Target CTACs were labeled with ⁵¹Cr for 1 hr and then washed. The ⁵¹Cr-labeled CTACs were then added at a concentration of 5×10^3 cells per well in 100 μ l of medium to the prediluted effector PBMCs and then incubated at 37°C for 4 hr. Supernatants were then collected and assayed for release of ⁵¹Cr activity with a γ counter (Medtronic, XXX). Spontaneous and maximum ⁵¹Cr release were also determined, and the percentage specific ⁵¹Cr release was calculated for each dilution of effector cells. The level of spontaneous specific killing activity determined in pre- and posttreatment PBMCs was used to assess the effect of treatment on NK activity.

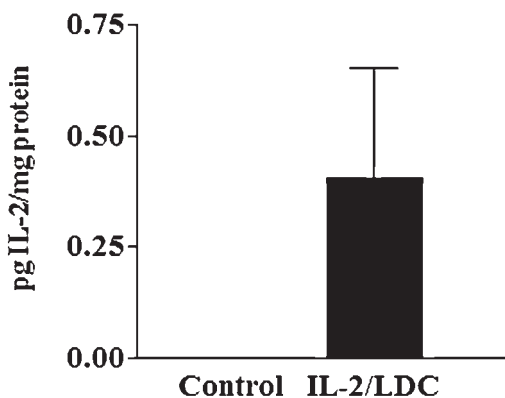


FIG. 1. IL-2 gene expression in lung tissues after intravenous gene delivery. Three beagle dogs were infused intravenously over 90 min with liposome–human IL-2 plasmid DNA complexes at a dose of 20 μ g/kg body weight. A fourth control animal received intravenous infusion with liposomes only. Twenty-four hours later, the dogs were humanely killed and tissues (lung, heart, liver, spleen, and kidney) were collected and analyzed for expression of human IL-2 by ELISA, as described in Materials and Methods. The mean IL-2 concentration (\pm SE) per milligram of tissue protein in lung tissues of the three dogs receiving IL-2 DNA was plotted, along with the IL-2 concentration present in lung tissues from the control dog. IL-2 expression was not detected in the other tissues examined (not shown).

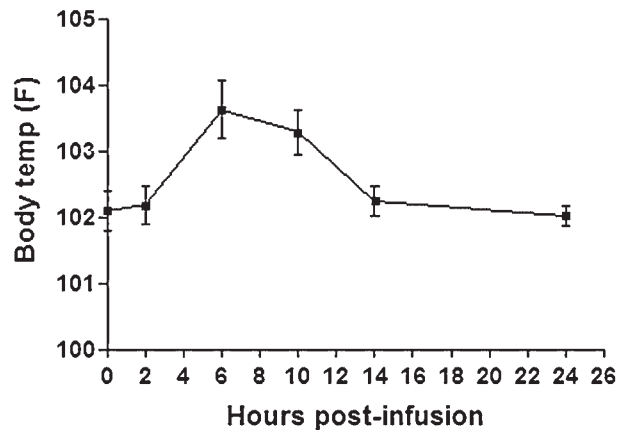


FIG. 2. Fever response after intravenous infusion of IL-2 LDCs in dogs. Ten dogs with osteosarcoma metastases were infused intravenously with liposome–canine IL-2 plasmid DNA complexes, body temperature measurements were obtained every 2 hr over a 24-hr period before and after the infusion, and the mean (\pm SE) body temperature was plotted for each time point. Each dog received an intravenous infusion over 90 min of liposome–IL-2 DNA complexes at a plasmid DNA dose of 20 μ g/kg body weight.

Uptake of labeled LDCs by leukocytes during intravenous infusion

Fluorescently labeled LDCs were infused in one dog to assess the uptake and distribution of LDCs by leukocytes in circulation during and after intravenous infusion. Fluorescently labeled cationic liposomes were prepared by addition of BODIPY-labeled cholesteryl (Molecular Probes, Eugene, OR) to unlabeled cholesterol and DOTIM at the time the liposomes were prepared. BODIPY-labeled cholesterol was added to unlabeled cholesterol at a 1:5 molar ratio and labeled and unlabeled cholesterol was added at a 1:1 molar ratio with DOTIM and then dried down. The labeled liposomes were rehydrated as described above, and then used to prepare LDCs. The labeled LDCs were infused at a DNA dose of 20 μ g/kg over a 90-min period, and serial blood samples were collected pretreatment and at 30, 60, 90, and 150 min. PBMCs were prepared and immunostained for detection of BODIPY-labeled complexes bound to lymphocytes or monocytes. Flow cytometric analysis was done as described above, with the exception that unlabeled CD4 and CD8 antibodies were used in these analyses and then stained with PE-conjugated anti-mouse immunoglobulin antibody.

RESULTS

Intravenous infusion of LDCs results in IL-2 gene expression in the lungs of dogs

Intravenous infusion of LDCs has been shown previously to preferentially transfect pulmonary tissues in mice (Liu *et al.*, 1997). To determine whether intravenous infusion of LDCs transfected tissues *in vivo* in dogs, we infused three normal dogs with LDCs at a dose of 20 μ g of DNA per kilogram body

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TABLE 1. EFFECTS OF INTRAVENOUS GENE DELIVERY ON HEMATOLOGIC PARAMETERS IN DOGS WITH OSTEOSARCOMA METASTASES^a

Parameter	Pretreatment cell count (\pm SD)	Posttreatment cell count (\pm SD)	Significance
Total WBC	7.48 (\pm 3.81)	5.16 (\pm 3.38)	$p = 0.01$
Neutrophils	3.20 (\pm 1.74)	2.57 (\pm 1.58)	$p = 0.19$
Lymphocytes	1.10 (\pm 0.43)	0.42 (\pm 0.28)	$p = 0.002$
Monocytes	0.39 (\pm 0.09)	0.39 (\pm 0.17)	$p = 0.95$
Platelets	318 (\pm 149)	181 (\pm 111)	$p = 0.003$

^aShown as the effect of intravenous IL-2 gene therapy on leukogram parameters. The acute effects of intravenous infusion of LDCs on leukogram parameters, including white blood cell counts (WBC), neutrophil counts (PMN), lymphocyte counts (lymph), and platelet counts, were assessed by comparing pretreatment and 24-hr posttreatment values for 10 dogs undergoing their first intravenous infusion of LDCs. Infusion of LDCs caused a significant decline in WBC, lymphocyte, and platelet numbers, but did not have a significant effect on neutrophil or monocyte numbers. Statistical comparisons of pre- and posttreatment values were done by paired *t* test. Data represent cells $\times 10^3/\mu\text{l}$ blood.

weight. These studies utilized a plasmid encoding the human IL-2 cDNA to facilitate detection of transgene expression *in vivo*. Twenty-four hours postinfusion, lung, liver, spleen, kidney, and lymph node tissues were collected and evaluated for human IL-2 expression by IL-2 ELISA. A fourth dog (control) was infused only with liposomes. Expression of human IL-2 was detected, albeit at low levels, in lung tissues of all three dogs infused with IL-2 LDCs, but not in the lungs of the control dog (Fig. 1). Expression of human IL-2 was not detected in any of the other tissues evaluated, in either the LDC-infused or control animals (data not shown). Microscopic lesions were not observed in the lung or other tissues of the three treated dogs (data not shown). Thus, intravenous infusion of low doses of plasmid DNA in dogs, relative to the amounts typically administered to rodents, produced low but detectable cytokine gene expression in lung tissues in dogs.

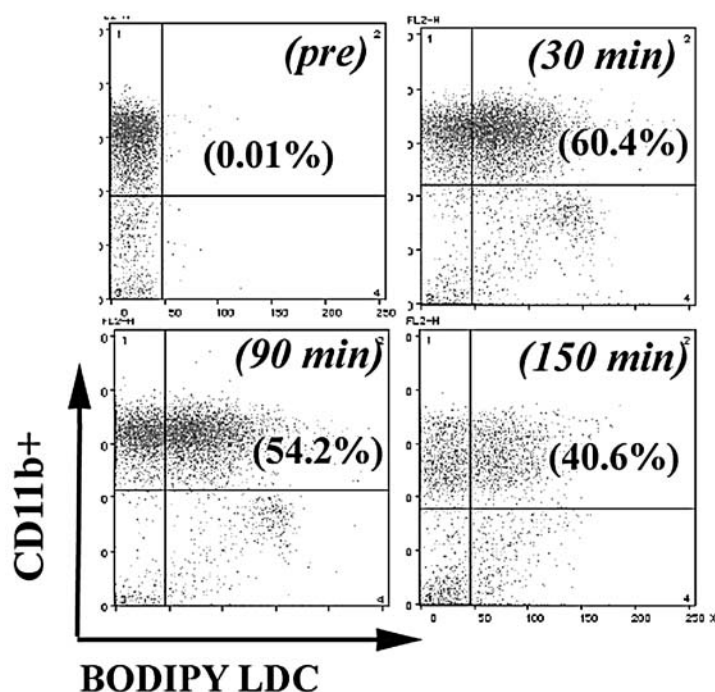
Intravenous infusion of LDCs elicits fever and transient lymphopenia and thrombocytopenia in dogs

The first five dogs enrolled in the study were treated intravenously on a weekly basis with increasing doses of IL-2 LDCs and carefully monitored during the first 24 hr postinfusion. At the initial dose of 5 μg of plasmid DNA per kilogram body weight dose, all five dogs developed mild fever but few other clinical signs (data not shown). At the subsequent 10- $\mu\text{g}/\text{kg}$ dose, the fever response was increased and transient thrombocytopenia became apparent, but the dogs were otherwise healthy. At the 20- $\mu\text{g}/\text{kg}$ dose, a marked fever response was observed (Fig. 2), along with lymphopenia and thrombocytopenia (Table 1), and the dogs became somewhat lethargic and anorectic. At the highest dose evaluated (40 $\mu\text{g}/\text{kg}$), one treated dog developed repeated vomiting and severe fever, necessitat-

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FIG. 3. Uptake of fluorescently labeled LDCs by PBMCs after intravenous infusion of IL-2 LDCs. The uptake of LDCs by peripheral blood mononuclear cells (PBMCs) was studied by infusion of fluorescently labeled LDCs. Liposomes labeled with the green fluorescent dye BODIPY (Molecular Probes) were infused over a 90-min period in a tumor-bearing dog. Serial blood samples were collected during the infusion (30 min), at the completion of the infusion (90 min), and 60 min after the infusion was completed (150 min). The PBMCs were immunostained for CD4, CD8, and CD11b expression with unconjugated primary antibodies followed by PE-conjugated secondary antibodies and analyzed by flow cytometry. By 30 min postinfusion, the majority of labeled LDCs were contained within the CD11b⁺ cell population, and this persisted at the completion of the infusion; the percentage of CD11b⁺ cells containing fluorescent complexes is shown in parentheses. Numbers in parentheses represent the percentage of CD11b⁺/BODIPY LDC⁺ cells. Few labeled LDCs were detected within either the CD4⁺ or CD8⁺ T cell populations (data not shown).



ing fluid support overnight. None of the dogs treated with LDCs at any dose developed clinically apparent respiratory abnormalities, including changes in either respiratory rate or effort. On the basis of these results, the 20- $\mu\text{g}/\text{kg}$ dose was determined to be the maximal safe and tolerated dose for dogs with cancer and this dose was used for all subsequent animals and treatments administered during the study.

Body temperature changes and leukogram changes were tabulated for a subset of the first 10 of 20 dogs treated at the 20- $\mu\text{g}/\text{kg}$ dose of IL-2 DNA. Body temperature increased beginning between 2 and 6 hr postinfusion, peaked between 6 and 10 hr, and then began to decline thereafter and was back to baseline by 24 hr postinfusion (Fig. 2). Twenty-four hours postinfusion, there was a significant decrease in total white blood cell count and lymphocyte counts (Table 1) whereas there was no significant change in numbers of neutrophils or monocytes. In addition, there was a significant decline in the platelet count in the first 24 hr postinfusion (Table 1). Cell counts returned to normal values by 1 week posttreatment (data not shown). At weeks 2 and 6 of the study, the overall numbers of lymphocytes (CD4^+ or CD8^+) were not significantly different from pretreatment values (data not shown). Moreover, the clinical responses elicited by intravenous infusions of LDCs were similar each time the infusions were repeated. These results are most consistent with the idea that the observed immune responses were mediated by activation of innate immunity by the liposome-DNA complexes, as has been reported previously in

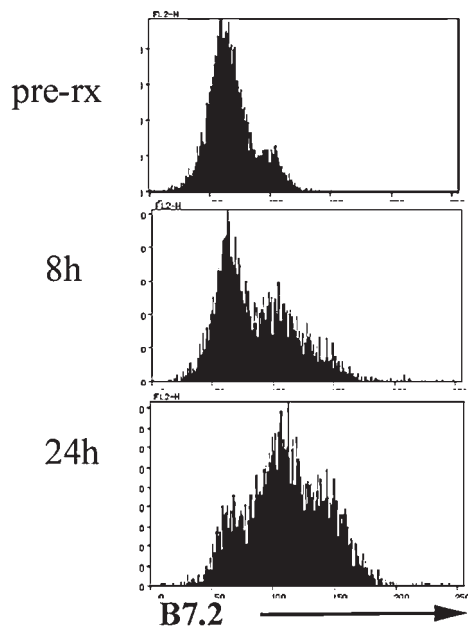


FIG. 4. Intravenous delivery of LDCs triggers upregulation of B7.2 expression by monocytes. Peripheral blood mononuclear cells were collected before and 8 and 24 hr postinfusion of IL-2 LDCs in a dog undergoing treatment. Cells were immunostained with an mAb to canine CD11b, and then with FITC-labeled anti-mouse IgG, followed by incubation with a PE-conjugated anti-B7.2 mAb. Expression of B7.2 by monocytes increased progressively during the first 24 hr after infusion of LDCs, indicative of monocyte activation.

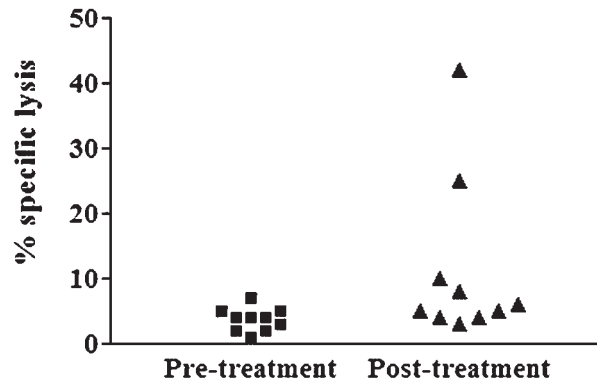


FIG. 5. Treatment with intravenous IL-2 LDCs elicits spontaneous NK cell activity. Spontaneous NK cell cytotoxicity was assessed before and 24 hr after infusion of IL-2 LDCs in 10 dogs with tumor metastases. Freshly collected PBMCs were used as effector cells in a 4-hr ^{51}Cr release assay, as described in Materials and Methods. Serial dilutions of effector cells were added to ^{51}Cr -labeled canine thyroid adenocarcinoma cells (CTACs) as targets in duplicate wells, starting at an effector-to-target ratio of 400:1 and diluting in 2-fold dilutions down to 12:1. The percentage specific lysis was calculated at each effector-to-target cell ratio. Specific lysis activity at an effector-to-target ratio of 400:1 was plotted for pre- and posttreatment PBMC samples for each dog.

rodent studies (Dow *et al.*, 1999a). Serum biochemical values were also monitored during the study and significant changes in any of 18 biochemical parameters monitored were not observed in treated dogs at either early or late time points posttreatment (data not shown). Thus, the major side effects of slow intravenous infusion of LDCs in dogs were fever and transient lymphopenia and thrombocytopenia in dogs, without injury to major organs including the liver or kidneys.

Liposome-DNA complexes extensively bound to monocytes during intravenous infusion

Fluorescently labeled liposomes were used to assess the distribution of LDCs in the bloodstream of dogs during slow intravenous infusion. Within 30 min of beginning the infusion, there was marked uptake of labeled LDCs by circulating CD11b^+ monocytes (Fig. 3). The percentage of monocytes containing BODIPY $^+$ LDCs remained relatively constant during and after the infusion. There was little uptake of labeled LDCs by circulating CD4^+ and CD8^+ T cells (data not shown), suggesting that LDCs were selectively bound by monocytes.

Intravenous infusion of liposome-DNA complexes elicits immune activation

Previous studies in mice by several different investigators have described potent activation of innate immunity by systemically administered LDCs, either with or without an encoded cytokine gene (Dow *et al.*, 1999a; Whitmore *et al.*, 1999; Norman *et al.*, 2000). We therefore assessed immune activation in dogs undergoing treatment with IL-2 LDCs. Within 24 hr of LDC infusion, strong upregulation of B7.2 expression (Fig. 4) and MHC class II expression (data not shown) by canine monocytes was observed, consistent with monocyte activation. In

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TABLE 2. NUMBERS OF TREATMENTS AND RESPONSE AND SURVIVAL TIMES IN 20 DOGS WITH OSTEOSARCOMA METASTASES TO THE LUNGS TREATED BY INTRAVENOUS GENE THERAPY USING LIPOSOME-DNA COMPLEXES^a

Dog	Response at 12 weeks	Total number of treatments	Survival time (days)
1	PD	2	14
2	PD	2	14
3	PD	3	34
4	PD	3	34
5	PD	1	35
6	PD	4	37
7	PD	5	56
8	PD	6	63
9	PD	8	72
10	PD	4	76
11	PD	7	88
12	SD	9	113
13	SD	13	116
14	PD	12	119
15	PD	9	132
16	PR	13	133
17	SD	14	185
18	CR	24	540
19	SD	26	866
20	PR	24	975

^aClinical data, including total number of treatments, tumor response at the end of 12 weeks of treatment, and overall survival times, were tabulated for the 20 dogs with osteosarcoma enrolled in this study. Treatment responses were classified as PD (progressive disease [$>50\%$ increase in tumor volume]), SD (stable disease [tumor size did not increase or decrease from pretreatment size by $>50\%$]), PR (partial regression [$>50\%$ decrease in tumor volume]), or CR (complete regression [all visible tumor gone]).

addition, mononuclear cells collected 24 hr postinfusion developed increased spontaneous cytolytic activity against MHC-mismatched target cells, compared with cytolytic activity from pretreatment mononuclear cells, although the overall difference in NK cell activity did not reach the level of statistical significance ($p = 0.07$) (Fig. 5). This cytolytic activity was most consistent with NK cell activation and killing, possibly mediated by LDC-induced IL-12 release (Dow *et al.*, 1999b). These data indicated that intravenous infusion of IL-2 LDCs in dogs was capable of triggering activation of innate immunity.

Clinical tumor responses to treatment with IL-2 LDCs

Twenty pet dogs with osteosarcoma metastatic to the lungs were entered into the clinical trial. Of these, only seven dogs completed the full 12-week treatment schedule (Table 2). Five dogs developed progressive tumor growth within the first month of beginning treatment and were humanely killed. Among the 14 dogs that survived beyond 1 month there were 3 clinical responses (either partial or complete tumor remission), and 4 dogs also had stable disease during the 12-week treatment period. One dog (dog 18 in Table 2) experienced a complete remission of multiple lung tumor nodules within the 12-week initial trial period (Fig. 6). This dog remained in remission for more than 500 days before succumbing to recurrent lung metastases. Two other dogs with stable disease survived for almost 900 and 1000 days. Three dogs received more than 20 infusions of IL-2 LDCs over a period of 1.5 to 3 years without experiencing cumulative toxicity or delayed adverse effects.

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Treatment with IL-2 LDCs prolongs overall survival times in dogs with lung metastases

The effect of treatment with IL-2 LDCs on overall survival times in dogs with stage IV osteosarcoma was assessed. Be-

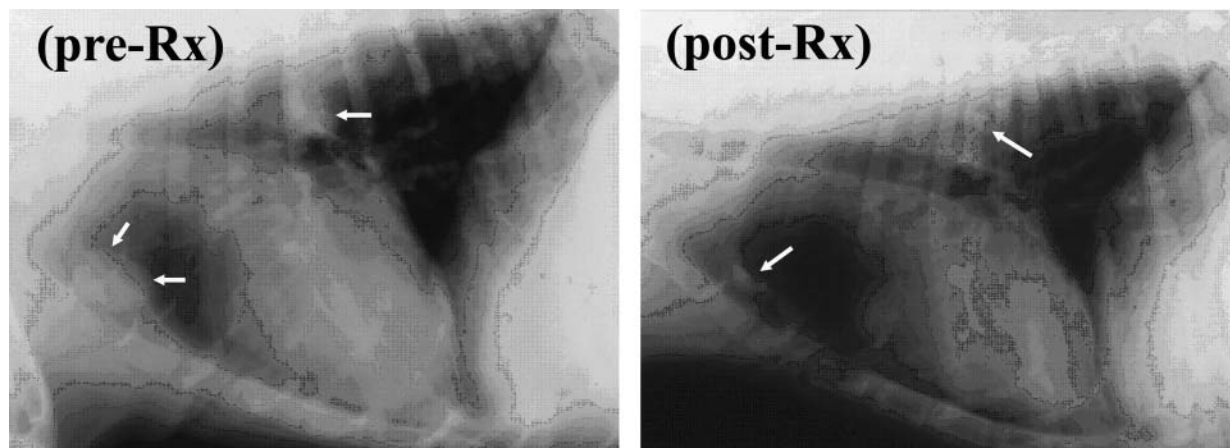


FIG. 6. Regression of pulmonary tumor metastases after intravenous infusion of IL-2 LDCs. Lung radiographs were obtained from a dog with osteosarcoma metastases to the lungs before treatment (left) and 18 weeks after treatment was initiated with IL-2 LDCs (right). Pretreatment there were numerous lung nodules throughout the lung fields, with several large metastases (arrows). Posttreatment, most of the smaller nodules had disappeared and there was a significant reduction in the size of the larger nodules (right). The lung nodules continued to disappear over time, although the largest nodule did not disappear entirely. This dog remained clinically normal for nearly 3 years, at which time the lung tumors recurred and the dog was killed.

cause this was a preliminary phase I study for safety and toxicity, a placebo-treated control group was not included in this study. Therefore, survival times for the 20 treated dogs in this study were compared with historical survival data generated from 40 dogs matched for tumor stage (stage IV disease, with lung metastases) and approximate age with the treatment population. The median survival time for the 40 untreated control dogs was 58 days, with a mean survival time of 81 days. The median survival time for the 20 dogs treated with IL-2 LDCs was 82 days, with a mean survival time of 228 days. When overall survival times were compared statistically by Kaplan–Meier survival analysis, there was a statistically significant increase ($p < 0.026$) in survival of treated dogs compared with untreated dogs (Fig. 7). Thus, treatment with IL-2 LDCs significantly improved outcome in dogs with metastatic tumors to the lungs, compared with historical control animals.

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DISCUSSION

In this report, we have extended our original observations of potent antitumor activity elicited by intravenous delivery of cytokine genes by LDCs in mouse tumor models to a more clinically relevant, spontaneous large animal tumor model. Here we show that repeated intravenous infusions of IL-2 encoding LDCs in dogs are well tolerated, capable of eliciting substantial systemic immune activation and able to prolong survival times even in dogs with advanced lung metastases. Importantly, these studies also demonstrate that cytokine-encoding LDCs are capable of producing gene expression in lung tissues *in vivo* at doses much lower than those typically employed in rodent intravenous gene therapy studies.

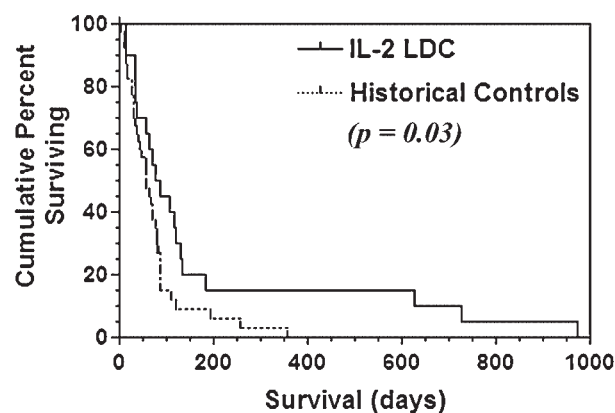


FIG. 7. Treatment with IL-2 LDCs prolongs overall survival times in dogs with stage IV osteosarcoma compared with disease-matched control animals. Survival curves were generated for 20 dogs treated in the present study and for historical data on 40 dogs with osteosarcoma of a similar disease stage (stage IV) that did not receive the intravenous gene therapy treatment. Overall survival times for the two patient populations were compared statistically by Kaplan–Meier analysis. The overall survival times for 20 dogs treated with IL-2 gene therapy were significantly greater ($p < 0.026$) than survival times for the control population.

Previous gene therapy approaches to cancer treatment *in vivo* have generally involved direct tumor transfection by viral or nonviral vectors. Such an approach, although technically simple, is limited primarily to those tumors where the tumor mass is accessible to needle injection (Nabel *et al.*, 1993; Clark *et al.*, 2000). In contrast, options for treatment of disseminated tumors by gene therapy are more limited. For example, repeated systemic gene delivery using viral vectors is not a viable option as severe toxicity can develop as a result of antivector immunity (Zhou *et al.*, 2004). However, immunotherapy of tumor metastases using nonviral gene therapy offers several potential advantages, including the combined effects of therapeutic genes (e.g., cytokine genes) with activation of innate immunity by the vector itself (Dow *et al.*, 1999b). Repeated administration is also possible because vector immunity to liposome–DNA complexes does not develop. In the case of lung tumors, intravenous injection of LDCs also results in selective targeting of gene delivery to the lungs and angiogenic endothelium (Li and Huang, 1997; Liu *et al.*, 1997; Thurston *et al.*, 1998; Krasnici *et al.*, 2003). Studies in mice have already clearly demonstrated the potency of LDCs in eliciting antitumor activity in animals with lung metastases (Dow *et al.*, 1999b; Whitmore *et al.*, 1999; Miyata *et al.*, 2001). However, such approaches have not been previously evaluated in large animal, spontaneous tumor models.

Extrapolating results of rodent studies directly to larger species is an empirical exercise at best. The issues that arise include dosing, rate of delivery, unexpected toxicities, and efficiency of gene expression. The studies reported here were therefore designed to address several of these questions. Dogs are a valuable animal model for cancer research because they develop several tumors that are biologically similar to those of their human counterparts (e.g., non-Hodgkin’s lymphoma, melanoma, and osteosarcoma). The kinetics of tumor growth and progression are also greatly accelerated in dogs relative to humans, making it possible to assess treatment outcomes in a much more realistic time frame (MacEwen, 1990; Vail and MacEwen, 2000). In addition, dogs are generally outbred, live in the same environment as humans, and often have similar drug distribution profiles. Osteosarcoma was selected for the studies reported here because it is a common tumor in dogs but, unfortunately, most dogs die of tumor metastases to the lungs within 1–2 years of diagnosis, even after amputation and aggressive adjuvant chemotherapy (MacEwen, 1990). Furthermore, the pet dogs enrolled in this study also did not have other available treatment options, as all had already failed conventional adjuvant chemotherapy.

The doses of LDCs used in this study were much lower than those typically used in rodent studies. For example, prior LDC gene delivery studies in mice have typically used plasmid DNA doses in the range of 2.5 to 5 mg/kg body weight. In contrast, dogs in this study were treated with a plasmid DNA dose of 20 μ g/kg body weight, a dose 125 to 250 times less than those used in mice. Gene expression could be detected in the lungs of animals treated with this dose, although the levels of expression were low. Several factors may account for differences in the efficiency of gene delivery between species. For one, the rate of administration in dogs was slow compared with the bolus injections typically administered to mice. This slow rate of infusion (90 min) was selected on the basis of studies in rab-

bits, where the slower rates of infusion consistently produced superior transfection of lung tissues (D. Liggitt, unpublished data). Moreover, in our experience larger animals will not tolerate bolus injection of liposome–DNA solutions. In addition, a fairly concentrated solution of LDCs was used (100 μg of DNA per milliliter), again on the basis of studies in rabbits demonstrating enhanced transfection efficiency in the lungs using more concentrated LDC solutions (D. Liggitt, unpublished data).

At LDC doses higher than 20 $\mu\text{g}/\text{kg}$, dogs began to manifest signs consistent with excessive immune activation (high fever, vomiting, and diarrhea). This response was not due to contaminating endotoxin, as the DNA used in these studies contained low levels of endotoxin. More likely, the side effects of LDC infusion were a manifestation of the systemic and rapid activation of innate immunity that is a prominent feature of intravenously administered LDCs (Dow *et al.*, 1999b; Whitmore *et al.*, 1999; Siders *et al.*, 2002; Wilson *et al.*, 2002). In support of this idea, we also observed similar effects (fever and leukopenia) in a dog treated with LDCs prepared with non-coding DNA, suggesting that the inflammatory response was a function of the delivery vehicle and not necessarily a manifestation of IL-2 gene expression (data not shown). The transient lymphopenia that was observed (Table 1) could probably also best be explained as a consequence of rapid systemic immune activation and margination of lymphocytes. In the case of decreased platelet counts, this response may have reflected the effects of immune activation and binding of platelets to LDC-activated endothelium, or of platelet aggregation by formation of complexes with the infused LDCs *in vivo*. Moreover, the relatively low levels of IL-2 gene expression achieved in lung tissues would be unlikely to result in high circulating levels of IL-2. For example, we were unable to detect upregulation of IL-2 receptor expression on lymphocytes of treated dogs after IL-2 LDC infusion (data not shown). Our prior studies in mice would suggest that activation of innate immunity by the LDCs may have provided a substantial portion of the antitumor activity observed here (Dow *et al.*, 1999b). However, we have also shown that antitumor activity can be significantly enhanced by the expression of the IL-2 gene in lung tissues, particularly in animals with more advanced lung metastases (Dow *et al.*, 1999a). Thus, it is likely that the effects of IL-2 gene delivery on lung tumors in dogs in this study reflected the effects of strong activation of innate immunity combined with the stimulatory effects of locally produced IL-2.

Several immunological mechanisms may account for induction of antitumor activity in dogs treated in this study. For example, activation of NK cell activity in treated dogs is consistent with rodent models, in which NK cells are the primary mediators of antitumor activity elicited by LDCs (Dow *et al.*, 1999b). However, cytotoxic T lymphocytes (CTLs) have also been shown to play an important role in mediating the antitumor effects of systemic gene therapy with LDCs in other mouse tumor models (Siders *et al.*, 2002). Cytotoxic T lymphocyte activity was not assessed in dogs in this study because of the lack of autologous tumor cell lines. Systemic release of interferons, especially IFN- γ and IFN- α , may also have played an indirect role in inhibiting tumor growth in the dogs. The upregulation of MHC class II and B7.2 expression by monocytes is consistent with the effects of systemic production of IFN- γ . In mouse

models, lack of IFN- γ production significantly inhibited the antitumor activity of LDCs (Dow *et al.*, 1999b). The induction of stable disease and prolonged disease remission in several treated dogs may also reflect ongoing angiogenesis inhibition. For example, we have observed that intravenous gene delivery using LDCs can inhibit angiogenesis in both mice and dogs (Dow, 2005).

In summary, we show here that repeated infusions of relatively low doses of IL-2 plasmid DNA in cationic liposomes can elicit significant antitumor activity in a large animal model of spontaneous tumor metastasis. The mechanism of antitumor activity is likely dependent on both gene-specific and nonspecific effects. These studies suggest that such an approach may be feasible for the treatment of tumor metastases to the lungs in humans. However, our studies also point out that LDCs are extremely potent immune activators and are in fact more active on a per-weight basis in large animals than in rodents. Therefore, clinical evaluation of intravenously administered LDCs in humans will need to include carefully designed dose titration studies with end points that assess activation of innate immunity. Nonetheless, intravenous gene delivery using LDCs may prove effective as part of a combined modality approach to cancer treatment, including gene therapy combined with conventional chemotherapy and/or radiation therapy.

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