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Intravenous Cytokine Gene Delivery by Lipid–DNA Complexes Controls the Growth of Established Lung Metastases

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ABSTRACT

Local expression of cytokine genes by *ex vivo* transfection or intratumoral gene delivery can control the growth of cutaneous tumors. However, control of tumor metastases by conventional nonviral gene therapy approaches is more difficult. Intravenous injection of lipid–DNA complexes containing noncoding plasmid DNA can significantly inhibit the growth of early metastatic lung tumors. Therefore, we hypothesized that delivery of a cytokine gene by lipid–plasmid DNA complexes could induce even greater antitumor activity in mice with established lung metastases. The effectiveness of treatment with lipid–DNA complexes containing the IL-2 or IL-12 gene was compared with the effectiveness of treatment with complexes containing noncoding (empty vector) DNA. Treatment effects were evaluated in mice with either early (day 3) or late (day 6) established lung tumors. Lung tumor burdens and local intrapulmonary immune responses were assessed. Treatment with either noncoding plasmid DNA or with the IL-2 or IL-12 gene significantly inhibited the growth of early tumors. However, only treatment with the IL-2 or IL-12 gene induced a significant reduction in lung tumor burden in mice with more advanced metastases. Furthermore, the reduction in tumor burden was substantially greater than that achieved by treatment with recombinant cytokines. Treatment with the IL-2 or IL-12 gene was accompanied by increased numbers of NK cells and CD8$^+$ T cells within lung tissues, increased cytotoxic activity, and increased local production of IFN-γ by lung tissues, compared with treatment with noncoding DNA. Thus, cytokine gene delivery to the lungs by means of intravenously administered lipid–DNA complexes may be an effective method of controlling lung tumor metastases.

OVERVIEW SUMMARY

Systemic delivery of cytokine genes to the lungs was evaluated as a treatment for metastatic lung cancer. Intravenous injection of lipid–DNA complexes containing the IL-2 or IL-12 gene significantly inhibited growth of both early and late established lung tumors, whereas complexes containing noncoding DNA only inhibited the growth of early tumors. Cytokine delivery by lipid–DNA complexes induced an increase in the number of NK cells and CD8$^+$ T cells in the lungs, along with increased IFN-γ production.

INTRODUCTION

CONTROL OF TUMOR METASTASES remains a major goal of cancer immunotherapy. Tumor transfection and immunization with immunostimulatory cytokine genes such as interleukin 2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-12 may be used to induce systemic antitumor immunity (Fearon *et al.*, 1990; Gansbacher *et al.*, 1990; Brunda *et al.*, 1993; Dranoff *et al.*, 1993; Lode *et al.*, 1998; Fernandez *et al.*, 1999). However, at present cytokine gene therapy of established tumors is generally limited to two ap-

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proaches: *ex vivo* transfection of autologous tumor lines (Fearon *et al.*, 1990; Gansbacher *et al.*, 1990; Brunda *et al.*, 1993; Dranoff *et al.*, 1993) or *in vivo* transfection by direct intratumoral injection of viral vectors or plasmid DNA (Nabel *et al.*, 1993; Plautz *et al.*, 1993; Parker *et al.*, 1996). Direct *in situ* transfection of lung tumor nodules has also been reported using ultrasound-guided injection, but this approach is limited to patients with discrete, large primary tumors (Roth *et al.*, 1996). Systemic immunotherapy with high doses of recombinant IL-2 produces objective tumor responses in approximately 15% of patients with advanced melanoma or renal cell carcinoma, but the treatment can also be associated with significant morbidity (Rosenberg *et al.*, 1994; Fyfe *et al.*, 1996). Thus, there is a need for a broadly applicable gene therapy approach to metastatic cancer that can produce systemic antitumor activity without the need for tumor cultures, direct tumor injection, or identification of tumor-specific antigens.

Intravenous injection of lipid–plasma DNA complexes is an efficient means of systemic gene delivery to the lungs (Zhu *et al.*, 1993; Liu *et al.*, 1995, 1997; McLean *et al.*, 1997; Song *et al.*, 1997; Templeton *et al.*, 1997). Intravenous injection of lipid–DNA complexes encoding the p53 gene was reported to reduce the growth and metastatic rate of human breast cancers in mice (Lesson-Wood *et al.*, 1995). Intravenous delivery of the angiostatin gene by means of lipid–DNA complexes was reported to control the growth of lung tumors in mice (Liu *et al.*, 1999). Intratracheal IL-12 gene delivery to the lungs using lipid–DNA complexes was also reported to inhibit significantly the growth of day-1 or day-3 established lung tumors (Blezinger *et al.*, 1999). Such an approach in humans would, however, be extremely limited because of the inability to safely deliver sufficient amounts of plasmid DNA.

As an alternative approach, intravenous cytokine gene delivery to the lungs using lipid–DNA complexes may be an effective method of treating metastatic lung cancer. We reported that intravenous injection of lipid–DNA complexes containing noncoding vector DNA induced pronounced immune activation and antitumor activity and controlled the growth of early established lung tumors (Dow *et al.*, 1999). However, we also observed that this so-called empty vector effect was relatively ineffective in controlling the growth of more advanced (>3 days after tumor injection) lung tumors. Thus, we sought to improve the antitumor efficacy of intravenous gene therapy by expressing a cytokine gene in the lungs using lipid–DNA complexes. The effects of IL-2 and IL-12 gene delivery on tumor growth and cellular immune responses were therefore compared with that of noncoding plasmid DNA. Antitumor efficacy was assessed by measuring the reduction in lung tumor burden after treatment of either early (day 3) or late (day 6) established lung tumors, using three different metastatic autologous tumor models in mice (melanoma, colon carcinoma, and fibrosarcoma).

We found that compared with treatment with lipid–DNA complexes containing noncoding DNA, treatment with DNA encoding the IL-2 or IL-12 gene induced significant inhibition of the growth of more advanced lung tumors. Treatment with the IL-2 or IL-12 gene was accompanied by increased pulmonary infiltration of natural killer (NK) cells and $CD8^{+}$ T cells, along with increased NK cell cytotoxicity and increased spontaneous local production of interferon γ (IFN-γ) by lung tissues. Thus, systemic, pulmonary-targeted cytokine gene delivery may be an effective approach to the treatment or prevention of cancer metastases to the lungs.

MATERIALS AND METHODS

Plasmid expression vectors

The cDNA for murine IL-2 was obtained from the American Type Tissue Collection (Rockville, MD) and was cloned into a eukaryotic expression vector that utilized the human cytomegalovirus (CMV) immediate-early promoter, an intron A sequence between the promoter and the start site, and the simian virus 40 (SV40) polyadenylation sequence (provided by J. Haynes, Heska Corporation, Ft. Collins, CO). The cDNAs for the p35 and p40 chains of the murine IL-12 gene were cloned by polymerase chain reaction (PCR) from normal mouse spleen cell cDNA. The two IL-12 chains were linked by an internal ribosomal entry site (IRES). The p40 gene was oriented upstream from the p35 gene and the resulting construct was cloned into the PCR3.1 vector (InVitrogen, San Diego, CA). This vector utilized the human CMV promoter and the bovine growth hormone polyadenylation sequence. Gene expression was confirmed by *in vitro* transfection and cytokine-specific enzyme-linked immunosorbent assay (ELISA) (PharMingen, San Diego, CA). In addition, bioassays were done to confirm production of biologically active cytokine. IL-2 was assayed by spleen cell proliferation and IL-12 activity was assayed by induction of IFN-γ release by spleen cells. The plasmid expression vector for luciferase was kindly provided by R. Debs (San Francisco, CA). The PCR 3.1 plasmid vector without an insert (empty vector) was used as a control for *in vivo* and *in vitro* transfection experiments.

Preparation of lipid–DNA complexes

Plasmid DNA was prepared by alkaline lysis from *Escherichia coli*, followed by polyethylene glycol (PEG) precipitation and ethanol and lithium chloride extraction, according to a previously published protocol (Liu *et al.*, 1997). The endotoxin content of the plasmid DNA was between 0.04 and 0.25 EU/μg of DNA. Cationic liposomes were prepared as multilamellar vesicles (MLVs) for *in vivo* use as described previously (Liu *et al.*, 1995). Briefly, 1,2-dioleoyl-3-trimethylammoniumpropoane (DOTAP; Avanti Polar Lipids, Alabaster, AL) and cholesterol (Sigma, St. Louis, MO) were mixed in a 1:1 molar ratio, dried down in round-bottom tubes, then rehydrated in 5% dextrose solution (D5W) by heating at 50°C for 6 hr. For *in vivo* gene delivery, lipid–DNA complexes were formed by mixing cationic liposomes with plasmid DNA at a ratio of 32 nmol of total DNA to 1.0 μg of DNA, at a final concentration of 100 μg of DNA per milliliter in D5W, as described previously (Templeton *et al.*, 1997).

Mice

Immune-competent mice were purchased from either Harlan-Sprague-Dawley (Indianapolis, IN) (C57BL6/J and ICR mice) or Jackson Laboratories (Bar Harbor, ME) (BALB/c mice) and were used between 10 and 14 weeks of age. C57BL/6 *nu/nu* mice were purchased from Jackson Laboratories. Proto-

cols for these experiments were approved by the Institutional Animal Care and Use Committee at the National Jewish Medical and Research Center (Denver, CO).

Tumor cell lines

The MCA-205 and YAC-1 cell lines were kindly provided by J. Routes (National Jewish Medical and Research Center). The F10 clone of the B16 melanoma cell line was provided by I. Fidler (M.D. Anderson, Houston, TX). The CT-26 colon carcinoma cell line was kindly provided by N. Restifo (National Cancer Institute, Bethesda, MD). Cells were maintained in modified Eagle's medium supplemented with 5% fetal bovine serum, penicillin, and streptomycin and were routinely treated with ciprofloxacin to assure mycoplasma-free conditions.

Assays for in vivo *gene expression*

In vivo gene expression in tissues was assessed by a luciferase reporter gene assay (Liu *et al*., 1997). Mice (four per treatment group) were injected intravenously with 100 μl of lipid–DNA complex in 5% dextrose in water (10.0 μg of total DNA injected), then sacrificed 24 hr later. Expression of cytokine genes in lung tissue was evaluated by the same protocol and DNA dosages. Tissues were harvested, homogenized in cell lysis solution (Analytical Luminescence Laboratories, Ann Arbor, MI), and the supernatants assayed for luciferase activity with a luminometer (Analytical Luminescence Laboratories) or for cytokine concentration with a cytokine-specific ELISA (PharMingen). The protein concentration of tissue lysates was quantitated by the bicinchoninic acid assay (Pierce, Rockford, IL) and the luciferase and cytokine concentrations were expressed per milligram of tissue protein.

ELISAs

The ELISAs for murine IL-2 and murine IL-12 p70 were purchased from PharMingen and were performed according to manufacturer directions. The ELISA for murine IFN-γ was performed with the XMG1.2 monoclonal antibody (MAb) as the capture antibody and biotinylated R4GA2 MAb as the detecting antibody. Concentrations of IFN-γ were determined by comparison with a standard curve generated with recombinant murine IFN-γ (R&D Systems, Minneapolis, MN).

Tumor injection and treatment schedule

Prior to tail vein injection, tumor cells were harvested by trypsinization to obtain single-cell suspensions and the number of viable cells was determined by trypan blue exclusion and cell counting with a hemocytometer. To establish lung tumors, mice were injected intravenously with 100 μl of a 2.5×10^6 cells/ml suspension of tumor cells. Tumor-bearing mice (four per treatment group) were treated beginning either on day 3 or day 6 after tumor cell injection. Treatments consisted of an intravenous injection of 100 μl of lipid–DNA complex, which delivered 10 μg of plasmid DNA per mouse. A second treatment was administered 7 days after the first, and the mice were sacrificed 7 days after the second injection for determination of lung tumor burden. For control mice, 100 μl of the diluent (5% dextrose in water) was injected at each treatment time point.

Flow cytometric analysis of lung mononuclear cells

To obtain intrapulmonary mononuclear cells, mice were euthanized and the heart was perfused with 10 ml of phosphate-buffered saline (PBS)–heparin solution to remove peripheral blood cells from lung tissues. Lung lobes were then carefully dissected free of bronchial-associated lymph node tissues, minced with scissors, and digested in a solution of collagenase (1.0 mg/ml), DNase (10 U/ml), and soybean-trypsin inhibitor (0.1 mg/ml) in tissue culture medium with 5% fetal bovine serum (all reagents were from Sigma). Lung tissues were incubated in the digestion solution for 1 hr at 37°C, and then triturated through a 15-gauge needle; the mononuclear cells were then isolated by Ficoll density gradient centrifugation, and the total number of viable mononuclear cells recovered from each lung was determined by manual counting.

Pulmonary mononuclear cells were analyzed with a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer, with analysis gates set by gating on unstained spleen lymphocytes. Between 10,000 and 30,000 gated events were analyzed for each cell population. For analysis of T cells, cells were dual-labeled with anti-$\alpha\beta$ T cell receptor (TCR) antibody (biotin–H57.597; PharMingen) and with antibodies to either CD4 (fluorescein isothiocyanate [FITC]–RM4-5; PharMingen) or CD8 (FITC–53-6.7; PharMingen). NK cells were dual-labeled with anti-NK 1.1 (biotin–PK136; PharMingen) and anti-CD3 (FITC–2C11). Monocytes in C57BL/6 mice were evaluated with anti-CR3 (biotin Mac-1; Pharmingen) and anti-IA^b (FITC–3F12.35; provided by J. Freed, National Jewish Medical and Research Center). Data were analyzed with Repromac software (True Facts Software, Seattle, WA) and the percentage of each cell type in the total lung mononuclear cell population was determined. The total number of each cell type was determined by multiplying the pulmonary mononuclear cell count by the percentage of each cell type.

Cytotoxicity assay

A standard 4-h ^{51}Cr release assay was used to quantitate cytotoxic activity in freshly isolated lung mononuclear cells from mice (four per treatment group), using YAC-1 cells, MCA-205 cells, or P815 cells as targets. Briefly, effector cells recovered from lung digests were added in decreasing concentrations to duplicate wells of a Linbro plate, to which was then added 5×10^3 target cells that had been previously labeled for 1 hr with ^{51}Cr. The plates were incubated at 37°C for 4 hr, then supernatants from each well were harvested and the amount of radioactive ^{51}Cr present was determined by automated γ counter. The percentage specific lysis was calculated as: observed ^{51}Cr release minus spontaneous ^{51}Cr release, divided by maximum ^{51}Cr release minus spontaneous ^{51}Cr release, times 100. The mean percentage specific lysis for each treatment group was calculated.

NK cell depletion

Tumor-bearing C57BL/6 mice were depleted of NK cells *in vivo* by a series of three intraperitoneal injections, 2 days apart, of 80 μg of IgG of PK136 MAb (kindly provided by J. Routes, National Jewish Medical and Research Center). This antibody is specific for the NK-1.1 surface antigen expressed on NK cells

(Yokoyama and Seaman, 1993). Control mice were injected with an equivalent amount of irrelevant isotype-matched MAb (Sigma). Injections of lipid–DNA complexes were begun 1 week later. The depleting antibody was administered again 2 days before the second DNA injection. In other experiments, NK cells were depleted by intraperitoneal injections of 50 μl of rabbit antiserum to asialo GM1 (Wako Bioproducts, Richmond, VA). These treatments eliminated NK cells (as assessed by flow cytometry) and splenic NK activity in resting and stimulated mice (data not shown).

Lung histology

For histologic evaluation of lung tissues, blood was removed from lung tissues by intracardiac perfusion, after which the lungs were insufflated with formalin by intratracheal cannulation. Tissues were paraffin imbedded, sectioned to a thickness of 4.0 μm, and stained with hematoxylin and eosin. Photomicrographs were made using an Olympus (Norwood, MA) photomicroscope.

Quantitation of lung tumor burden

For quantitation of lung tumor burden, lungs were infused intratracheally with India ink solution, then bleached in Fekete's solution, as described previously (Wexler, 1966). The total number of lung nodules present on the surface of the lungs of each mouse was determined by manual counting under a dissecting microscope.

Statistical analysis

Statistical differences between multiple treatment groups were compared by the Tukey–Kramer multiple comparisons procedure. Results were considered significant for $p < 0.05$. For comparisons between two treatment groups, the Student t test was used. Analyses were done using SAS.JMP statistical software (SAS, Cary, NC).

RESULTS

Gene expression in lung tissues after intravenous injection of lipid–DNA complexes

Mice were injected intravenously with lipid–DNA complexes encoding the luciferase reporter gene and gene expression in various tissues was measured 24 hr later. High levels of gene expression were detected in lung tissues, but not in heart, spleen, or liver tissues. Thus, the intravenous injection of lipid–DNA complexes confined gene expression primarily to lung tissues (Fig. 1A). In addition, the IL-2 gene was expressed efficiently in pulmonary tissues after injection of DNA (Fig. 1B). Expression of IL-12 p70 in lung tissues was below the level of sensitivity of the ELISA, but bioassays confirmed that biologically active IL-12 was produced by the IL-12 gene expression vector (data not shown). The duration of pulmonary gene expression was relatively transient and reporter gene expression decreased to low lev-

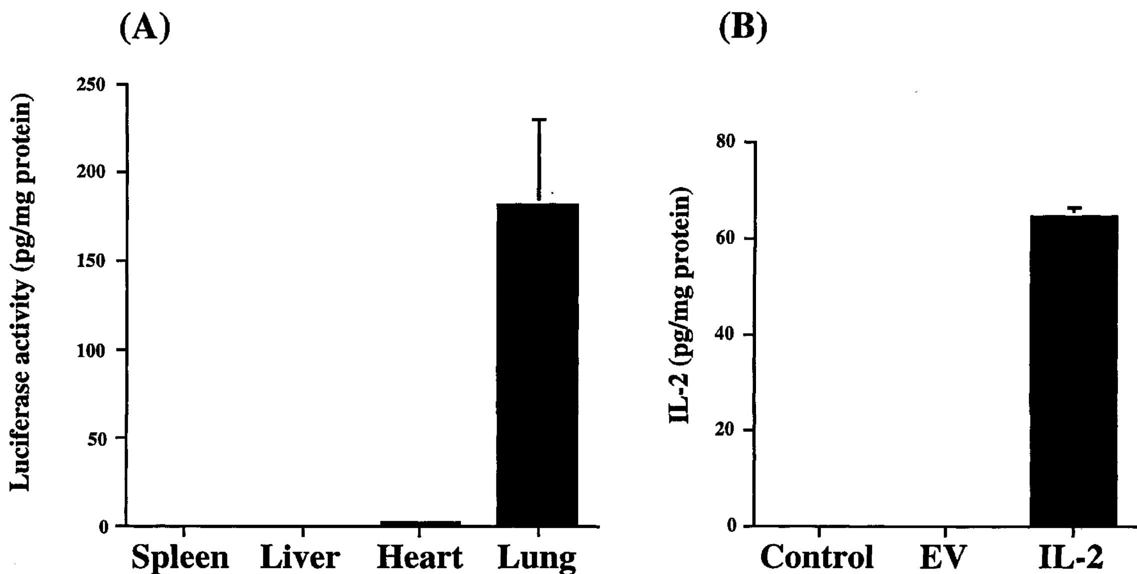

FIG. 1. Gene expression in pulmonary tissues after intravenous administration of lipid–DNA complexes. The efficiency of gene delivery and expression in pulmonary tissues was assessed by luciferase reporter gene assay and by cytokine ELISA. In (**A**), mice (four per group) were sacrificed 24 hr after lipid–DNA complex injection and the luciferase concentration in lung, heart, spleen, and liver tissues was determined as described in Materials and Methods. Control mice were injected with an equal amount of noncoding (empty vector) DNA. Results were expressed as the mean (±SE) luciferase protein concentration per milligram of tissue protein. Similar results were obtained in three additional experiments. Pulmonary concentrations of IL-2 were measured in mice after intravenous injection of lipid–DNA complexes containing the murine IL-2 plasmid DNA (**B**). Control mice were injected either with diluent (control) or with empty vector plasmid DNA (EV). The concentration of IL-2 in pulmonary tissues harvested 24 hr postinjection was quantitated by IL-2 ELISA. Results were expressed as the mean IL-2 concentration (picograms) per milligram of lung tissue protein (±SE).

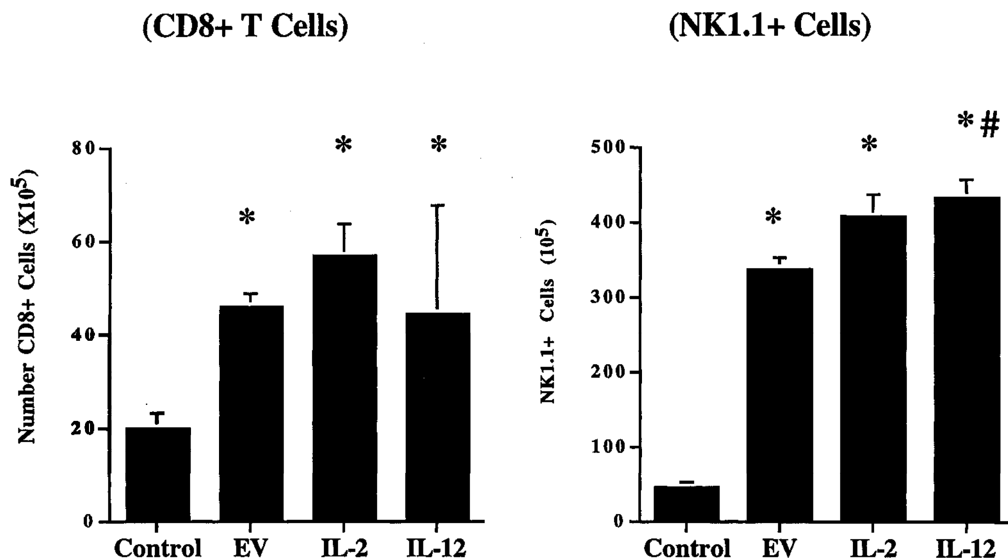

FIG. 2. Intravenous injection of plasmid–DNA complexes induces a large increase in intrapulmonary $CD8^+$ T cells and NK cells. Mice with established MCA-205 pulmonary metastases were injected intravenously with lipid–DNA complexes containing either empty vector DNA (EV), IL-2 DNA, or IL-12 DNA. Three days later, lung mononuclear cells were harvested by collagenase digestion, separated by Ficoll density gradient centrifugation, counted, and then analyzed by flow cytometry as described in Materials and Methods. The total number of $CD8^+$ T cells and NK cells per lung was determined for each animal, on the basis of the percentage of each cell type present in the lung mononuclear cell population as determined by flow cytometry. The results of two independent experiments were pooled and the mean number ($\pm$SE) of intrapulmonary $CD8^+$ T cells (*left*) and NK cells (*right*) for each treatment group was determined. Injection of lipid–DNA complexes induced a significant increase in both intrapulmonary $CD8^+$ T cells and NK cells, compared with untreated control animals with tumors. Injection of IL-12 DNA also induced a significant increase in NK cells compared with injection of empty vector DNA. Similar results were observed in mice with B16.F10 lung tumors. ($^*p < 0.05$ compared with control animals; $^\#p < 0.05$ compared with empty vector-treated animals).

els in pulmonary tissues by day 7 postinjection (data not shown).

NK cells and $CD8^+$ T cells accumulate in lung tissues after intravenous injection of lipid–DNA complexes

Flow cytometry was used to quantitate the changes in lung cellularity. Mononuclear cell suspensions were prepared from enzymatically digested lung tissues of mice with established pulmonary tumor metastases (either MCA-205 or B16 tumors). The cells were obtained either 3 or 6 days after injection of lipid–DNA complexes. Control mice were injected with diluent (5% dextrose in water). There was a significant, two- to threefold increase ($p < 0.05$) in the number of $CD8^+$ T cells infiltrating lung tissues in all three groups of treated mice, compared with control mice (Fig. 2, left). The largest increase in $CD8^+$ T cells was observed in mice treated with the IL-2 gene. Mice injected with the IL-2 gene also had a larger increase in $CD8^+$ T cells ($p = 0.06$) than mice injected with lipid–DNA complexes containing empty vector (EV) DNA.

The most striking effect of lipid–DNA complex injection was the 8- to 10-fold increase in the number of pulmonary interstitial NK-1.1^+ cells in treated mice compared with control mice, both at 3 days postinjection (Fig. 2, right) and 6 days postinjection (data not shown). Mice injected with the IL-12 gene had a significant increase ($p = 0.04$) in intrapulmonary NK cells compared with mice injected with empty vector DNA. The number of $CD4^+$ T cells was increased approximately 30% in all three groups of treated mice, compared with control animals (data not shown). The percentage of intrapulmonary monocytes was increased slightly in treated mice compared with controls, whereas the percentage of B cells was unchanged. (data not shown). Thus, intravenous injection of lipid–DNA complexes induced a large and sustained increase in both intrapulmonary NK cells and $CD8^+$ T cells in the lungs of tumor-bearing mice.

Pulmonary mononuclear cells in mice injected with lipid–DNA complexes express high levels of NK cell activity

Functional activation of lung mononuclear cells was assessed in a 4-hr NK cell cytotoxicity assay. High levels of spontaneous cytotoxicity against the NK cell-sensitive YAC-1 cell line were observed in mononuclear cells recovered from lung tissues of treated C57BL/6 mice 48 hr after intravenous injection of lipid–DNA complexes (Fig. 3A). Control animals had low background levels of cytotoxic activity. The cytotoxic activity of lung mononuclear cells was not MHC restricted, as revealed in studies using MHC-mismatched target cells (data not shown), consistent with NK cell-mediated cytotoxicity. High levels of NK cell cytotoxic activity against MCA tumor cells was also observed (Fig. 3B). Injection of lipid–DNA complexes encoding either the IL-2 or IL-12 gene induced higher levels of cytotoxic activity than did injection of empty vector DNA. The cytotoxic activity of lung mononuclear cells peaked 24–72 hr postinjection and declined over the next 3–4 days (data not

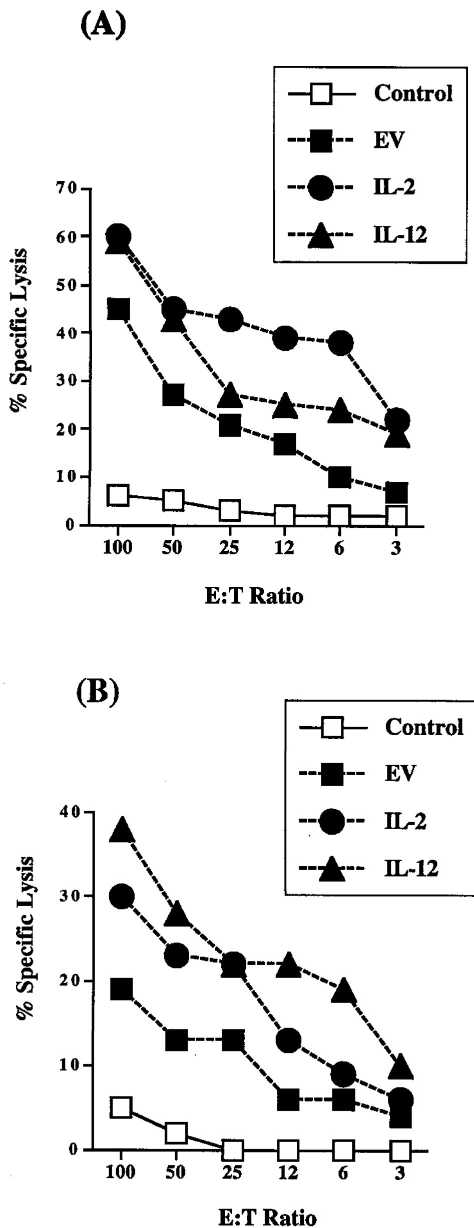

FIG. 3. Intravenous injection of lipid–DNA complexes induces high levels of NK cell activity in lung tissues. The effect of pulmonary gene delivery on the functional activity of lung mononuclear cells was assessed in a 4-hr postinjection from mice (four per group) injected with either diluent or lipid–DNA complexes containing empty vector DNA, IL-2 DNA, or IL-12 DNA. The lung mononuclear cells were purified as described in Materials and Methods and were assayed immediately after isolation. Spontaneous cytotoxic activity against either YAC-1 target cells **(A)** or MCA-205 target cells **(B)** was measured in a 4-hr chromium release assay. The mean percentage specific lysis of target cells at decreasing effector-to-target (*E*:*T*) ratios was determined for each treatment group and plotted. High levels of NK cell cytotoxic activity were exhibited by lung mononuclear cells isolated from treated mice, compared with control animals. In addition, the level of cytotoxic activity in the lungs of mice injected with IL-2 or IL-12 DNA was higher than in mice treated with empty vector DNA. Similar results were observed in two additional experiments.

shown). Thus, intravenous injection of lipid–DNA complexes induced functional activation of intrapulmonary NK cells, and local expression of either the IL-2 or IL-12 gene further enhanced the cytotoxic activity of intrapulmonary NK cells.

Mononuclear cell infiltration into lung tumors after intravenous injection of lipid–DNA complexes

The effect of intravenous gene delivery on cellular responses to established lung tumor nodules was evaluated histologically. Lung tissues from mice with day 6 established B16 pulmonary tumor metastases were harvested 3 days after intravenous injection of DNA (Fig. 4). Tumor nodules in sham-treated mice contained minimal peritumoral or intratumoral cellular infiltrates, and the surrounding lung parenchyma was normal (Fig. 4a and b). In contrast, there was extensive peritumoral and intratumoral infiltration of mononuclear cells in the lungs of treated mice (Fig. 4c and d). There was also a mild and diffuse increase in overall cellularity of pulmonary parenchymal tissues of treated mice. Similar results were also observed in mice with MCA-205 lung tumors (data not shown). The histologic responses were qualitatively similar in mice treated with lipid–DNA complexes containing either empty vector DNA or IL-2 or IL-12 DNA (data not shown).

Treatment of mice with established lung tumor metastases: Effects of treatment timing and cytokine genes on lung tumor burdens

The effect of treatment on the tumor burden in mice with established metastatic lung tumors was assessed in three different tumor metastasis models (Fig. 5). MCA-205 (fibrosarcoma) or B16.F10 (melanoma) tumors were established in the lungs of C57BL/6 mice by intravenous injection of 2.5×10^5 tumor cells per mouse, and CT26 (colon carcinoma) tumors were established similarly in BALB/c mice. After the tumors were injected, the mice were treated by systemic gene delivery either on day 3 after tumor injection (Fig. 5, Day 3 Rx) or on day 6 after tumor injection (Fig. 5, Day 6 Rx). The treatment was repeated once 7 days later and the animals were sacrificed 7 days after the second treatment. At this time (17 to 21 days after tumor injection) most of the control mice were beginning to exhibit signs of advanced tumor growth. The pulmonary tumor burden was quantitated by counting the number of tumor nodules, as described previously (Wexler, 1966).

Intravenous injection of lipid–DNA complexes induced a marked and significant decrease in the tumor burden in the lungs of all mice with early established lung metastases (day 3 after tumor injection), in all three tumor models evaluated (Fig. 5A–C). Treatment with empty vector DNA, as well as IL-2 or IL-12 DNA, induced a significant antitumor effect. The reduction in the number of tumor nodules in treated versus control mice was highly significant ($p < 0.01$ to $p < 0.001$) in mice with all three types of tumors. Overall, for all three tumor types injection of lipid–DNA complexes containing empty vector DNA induced a 75% decrease in the number of tumor nodules, whereas injection of IL-2 DNA induced an 85% decrease and IL-12 DNA induced a 94% decrease in the number of tumor nodules.

The effect of treatment on more advanced pulmonary metastases was also evaluated. When treatment was delayed until day

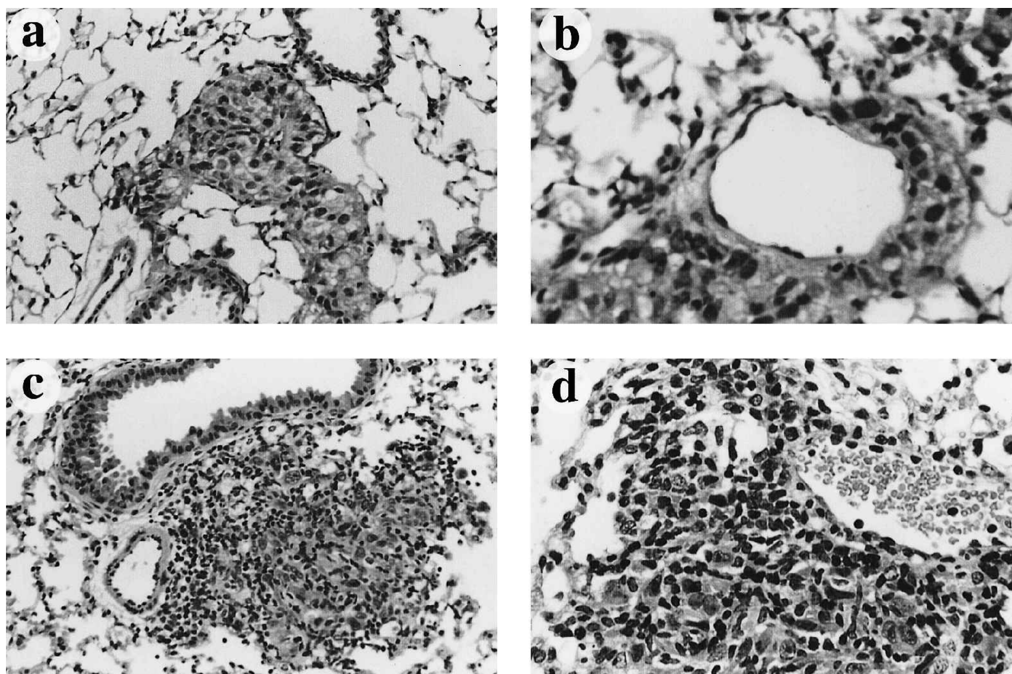

FIG. 4. Pulmonary gene delivery induces intratumoral and peritumoral mononuclear cell infiltration of lung tumor metastases. Mice with established B16.F10 lung tumors were injected intravenously with either diluent (control) or lipid–DNA complexes. Seven days postinjection, lung tissues were collected, fixed in formalin, sectioned, stained with H&E, and photographed. There was minimal inflammation around or within lung metastases in control mice (**a** and **b**). In contrast, marked mononuclear cell infiltration was present in and around a pulmonary tumor nodule in the lung of a mouse treated with lipid–DNA complexes (**b** and **d**). Mononuclear cells were particularly numerous in tumor tissues adjacent to blood vessels **(d)**. These mononuclear cell infiltrates were present in or around nearly all tumor nodules present in the lungs of treated mice (data not shown). Similar changes were observed in lung tissues obtained on either day 3 or day 6 postinjection, in mice with either B16 or MCA tumors, and in mice treated with empty vector, IL-2, or IL-12 genes. Original magnification: (**a** and **c**) ×55; (**b** and **d**) ×110.

6 after tumor injection, the antitumor effects induced by injection of lipid–DNA complexes containing empty vector DNA largely disappeared (Fig. 5A–C). Thus, there was no significant difference in the number of lung tumor nodules in mice treated with empty vector DNA on day 6 after tumor injection, compared with control animals, in any of the three tumor models. However, there was a significant reduction in lung tumor burden in mice treated with either IL-2 or IL-12 DNA, compared with either control mice or mice treated with empty vector DNA. Overall, there was still a 49 and 54% reduction in lung tumor burden in mice treated with IL-2 or IL-12 DNA, respectively, compared with control mice. Thus, tumor size and maturity appeared to be a major determinant of the responsiveness of tumors to treatment with lipid–DNA complexes containing empty vector DNA. The additional antitumor activity conferred by local expression of the IL-2 or IL-12 gene was sufficient to inhibit tumor growth, even in animals with more advanced tumors.

Injection of lipid–DNA complexes induces significant antitumor activity in mice lacking T cells

Since both T cell and NK cell numbers were elevated in the lungs of treated mice, experiments were done to assess the relative contribution of each cell type to control of tumor growth. First, C57BL/6 nude mice (*nu/nu*) were evaluated to determine the contribution of T cells to the antitumor effect. The number of MCA-205 lung tumor nodules was significantly reduced in *nu/nu* mice after treatment with either empty vector DNA ($p = 0.026$) or IL-2 DNA ($p = 0.003$), compared with untreated control *nu/nu* mice (Fig. 6). Nonetheless, the reduction in tumor burden in treated *nu/nu* mice was still less than in wild-type mice (66 versus 88% reduction in overall lung tumor burden). Furthermore, the lung tumor burden in wild-type mice treated with IL-2 DNA was significantly less ($p = 0.005$) than in *nu/nu* mice treated with IL-2 DNA. Thus, although the majority of the antitumor activity induced by intravenous injection of lipid–DNA complexes was T cell independent, T cells did exert some activity in controlling tumor growth, particularly after injection of IL-2 DNA.

Effect of NK cell depletion on antitumor activity

The role of NK cells was investigated by *in vivo* depletion of NK cells prior to treatment with lipid–DNA complexes. Mice were depleted of NK cells by injection of the PK136 MAb, which recognizes the NK-1.1 surface antigen on NK cells (Yokoyama and Seaman, 1993). Antibody-mediated depletion of NK cells in C57BL/6 mice with MCA-205 tumors significantly reduced the antitumor activity induced by injection of either empty vector DNA or IL-2 DNA (Fig. 7). The tumor burden in the lungs of NK cell-depleted mice treated with either

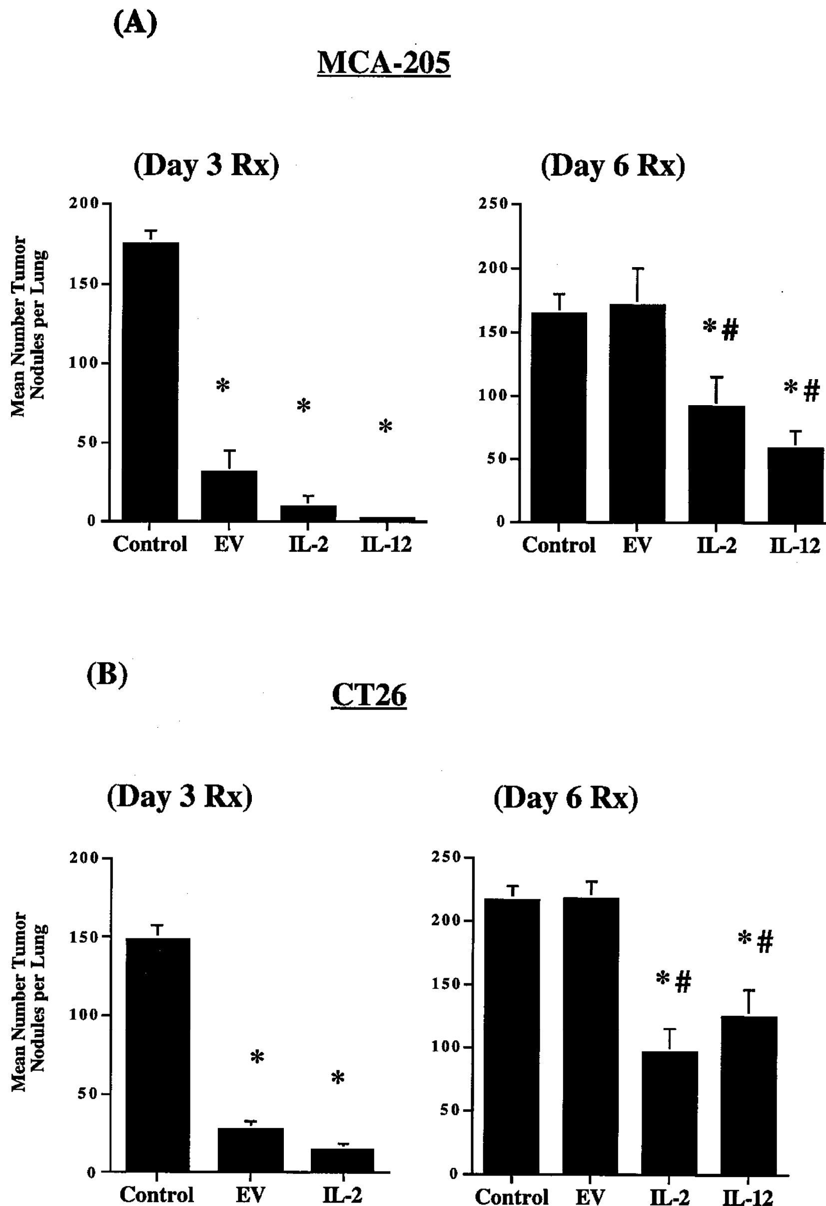

FIG. 5. Reduction in lung tumor burden by intravenous administration of lipid–DNA complexes: effects of treatment timing and administration of cytokine genes. Tumors were established in mice by intravenous injection of 2.5×10^5 tumor cells per mouse, using one of three different tumor cell lines: **(A)** MCA, **(B)** CT26, or **(C)** B16. Three days (*left*) or 6 days (*right*) after tumor injection, mice (four per treatment group) were injected intravenously with lipid–DNA complexes, as described in Materials and Methods. Control mice were injected with diluent only; and other groups of mice were injected with lipid–DNA complexes containing empty vector DNA (EV), murine IL-2 DNA, or murine IL-12 DNA. The injections were repeated 7 days later, and the mice were sacrificed 7 days after the second injection. The number of tumor nodules in the lungs of each mouse was determined as described in Materials and Methods and the mean number of tumor nodules per treatment group (± SE) was plotted. Each experiment was repeated at least once. Statistical differences between treatment groups were determined by the Tukey–Kramer multiple comparisons procedure, using pooled data from two independent experiments. When mice were treated on day 3 after tumor injection, treatment with lipid–DNA complexes prepared with either empty vector, IL-2, or IL-12 DNA produced a significant decrease ($p < 0.01$ to $p < 0.001$) in the number of tumor nodules compared with control mice, in mice with any of the tumor types evaluated. However, when the start of treatment was delayed until day 6 after tumor injection, treatment with lipid–DNA complexes containing empty vector DNA did not induce a significant decrease in tumor burden in any of the three tumor models evaluated. However, there was a significant reduction in lung tumor burden in both IL-2 and IL-12 DNA-treated mice in all three tumor models, compared with either control mice or mice treated with empty vector DNA. ($^*p < 0.05$ compared with control mice; $^\#p < 0.05$ compared with empty vector-treated mice.)

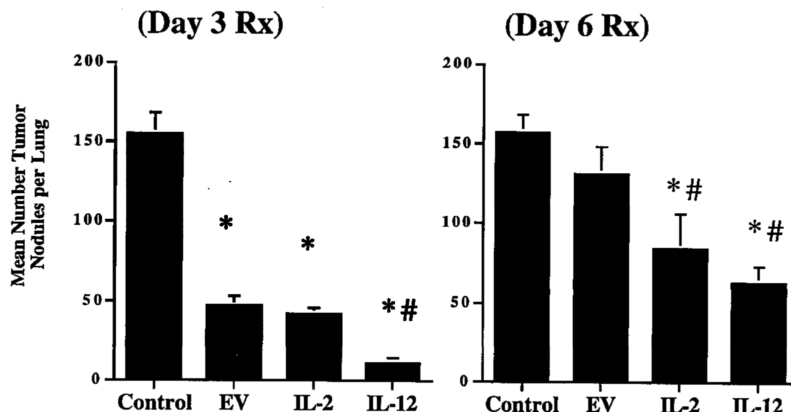

FIG. 5. (*continued*)

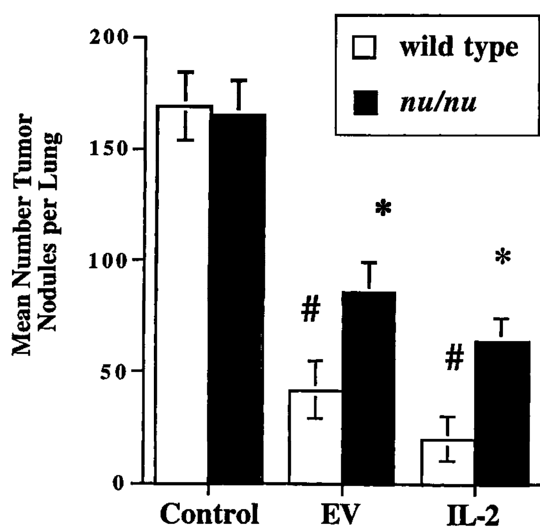

FIG. 6. Effects of injection of lipid–DNA complexes on tumor burden in nude mice. C57BL/6 *nu/nu* and wild-type mice (four mice per group) with MCA-205 lung tumor metastases were treated on day 3 after tumor injection with lipid–DNA complexes containing either empty vector DNA or IL-2 DNA. The treatment was repeated once 7 days later and the mice were sacrificed 7 days after the last treatment. The lung tumor burden was quantitated and the mean number of tumor nodules per lung (±SE) was plotted for each group. The lung tumor burden in both wild-type (open bars) and *nu/nu* mice (filled bars) was significantly reduced ($p < 0.05$) compared with their respective controls by injection of either empty vector or IL-2 DNA. In addition, the lung tumor burden in wild-type mice treated with IL-2 DNA was significantly less than in *nu/nu* mice treated with IL-2 DNA. This experiment was repeated once with similar results. ($^*p < 0.05$, compared with *nu/nu* control mice; $^{\#}p < 0.05$ compared with similarly treated *nu/nu* mice.)

empty vector or IL-2 DNA was not significantly different ($p > 0.05$) than the tumor burden in control, NK cell-depleted mice. Injection of lipid–DNA complexes elicited a significant reduction in the number of lung tumor nodules in mice pretreated with an irrelevant, isotype-matched antibody. Taken together, these results indicate that NK cells played a primary role in mediating the antitumor activity induced by systemic injection of lipid–DNA complexes.

Pulmonary expression of IL-2 or IL-12 genes increases local IFN-γ production

NK cells and $CD8^+$ T cells can mediate antitumor activity directly, or indirectly by release of cytokines, particularly IFN-γ. Therefore, we measured release of IFN-γ from lung tissues 48 hr after injection of lipid–DNA complexes (Fig. 8). Lung tissues were collected, then minced and cultured under sterile conditions in complete medium for an additional 18 hr. The supernatants were harvested and assayed for release of IFN-γ, using an ELISA. At 24 hr postinjection, complexes of lipid and empty vector DNA induce substantial release of IFN-γ by lung tissues, but this effect quickly wanes (Dow *et al.*, 1999). Therefore, when measured at 48 hr posttreatment, injection of lipid–DNA complexes composed of empty vector DNA elicited only a modest increase in IFN-γ release beyond control levels (Fig. 8). In contrast, lung tissues from mice injected with either IL-2 or IL-12 DNA released significantly more IFN-γ than either control mice or mice injected with empty vector DNA at 48 hr postinjection. This finding is important because we found that IFN-γ was a major mediator of the antitumor activity induced by intravenous injection of lipid–empty vector DNA complexes (Dow *et al.*, 1999). Thus, sustained exposure to locally produced IFN-γ may account for the increased effectiveness of IL-2 or IL-12 gene delivery for treatment of advanced tumors, when compared with injection of empty vector DNA.

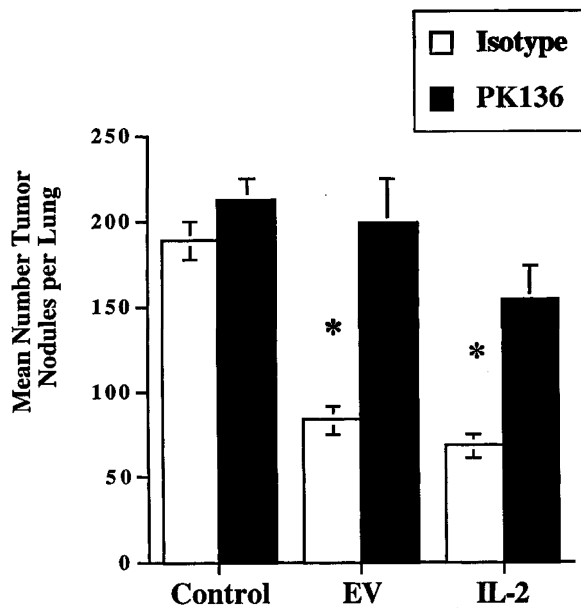

FIG. 7. Effect of NK cell depletion on response to treatment with lipid–DNA complexes. C57BL/6 mice (four per treatment group) with MCA-205 lung tumors were treated with intraperitoneal injections of PK136 MAb to deplete NK cells prior to treatment with lipid–DNA complexes, as described in Materials and Methods. Control mice were treated with an isotype-matched antibody (isotype, open bars). Treatment with lipid–DNA complexes was initiated on day 3 after tumor injection, repeated 7 days later, and the mice were sacrificed and the lung tumor burden quantitated 7 days after the last injection. The mean number of tumor nodules in NK cell-depleted mice (solid bars) treated with empty vector or IL-2 DNA was not significantly different from the number of tumor nodules in control, NK cell-depleted mice. The number of tumor nodules in NK-depleted mice treated with lipid–DNA complexes was, however, significantly greater ($p = 0.02$) than in treated mice that were administered an irrelevant isotype-matched antibody. Similar results were obtained in one additional NK cell depletion experiment. ($^{*}p < 0.05$ compared with NK cell-depleted mice treated similarly.)

DISCUSSION

Intravenous injection of lipid–DNA complexes has been shown to be a powerful stimulus for induction of immune activation and antitumor activity (Dow *et al.*, 1999). Here, we have extended those observations and now show that administration of a T cell- and NK cell-stimulatory cytokine (either IL-2 or IL-12) can significantly augment the antitumor effects of DNA–lipid complexes. This enhanced antitumor efficacy was most apparent when mice with more advanced tumors were treated, since under those conditions the so-called empty vector effect was greatly diminished. The enhanced antitumor efficacy of intravenous cytokine gene delivery was demonstrated in three different tumor models, using two different strains of mice.

The nonspecific antitumor effects of lipid–DNA complexes had been observed previously by other investigators, after either intravenous or direct intratumoral administration (Lesoon-Wood *et al.*, 1995; Parker *et al.*, 1996). Bacterial DNA is immunogenic in mammals and capable of triggering activation of innate immune responses, including activation of NK cells (Tokunaga *et al.*, 1984; Krieg *et al.*, 1995; Ballas *et al.*, 1996; Klinman *et al.*, 1996; Pisetsky, 1996; Chace *et al.*, 1997). Thus, many of the pulmonary cellular changes induced by intravenous injection of lipid–DNA complexes containing empty vector DNA were mediated by immune activation by plasmid DNA, and these responses were significantly enhanced by formation of the lipid–DNA complex (Dow *et al.*, 1999).

Interleukin 2 and IL-12, both of which elicited strong antitumor activity in our system, have potent NK cell- and T cell-stimulatory properties. Our results are therefore consistent with the idea that the major function of the cytokine gene when delivered using lipid–DNA complexes is to enhance and/or prolong the function of NK cells and T cells that are initially activated by the lipid–DNA complexes themselves. In support of this idea, we observed that systemic delivery of the IFN-γ gene (another NK cell-stimulatory cytokine) also induced strong antitumor activity, whereas systemic delivery of the GM-CSF gene, which lacks NK cell-stimulatory activity, did not (data not shown).

Intravenous cytokine gene delivery achieved a much greater reduction in lung tumor burden in the present study than did repeated administration of high doses of either IL-2 or IL-12 protein in a previous study, using the same tumor model (Irvine *et al.*, 1996). Thus, intravenous cytokine gene therapy is not simply an alternative method of cytokine delivery. Rather, the effectiveness of the lipid–DNA-mediated cytokine gene delivery approach reflects the combined effects of potent immune

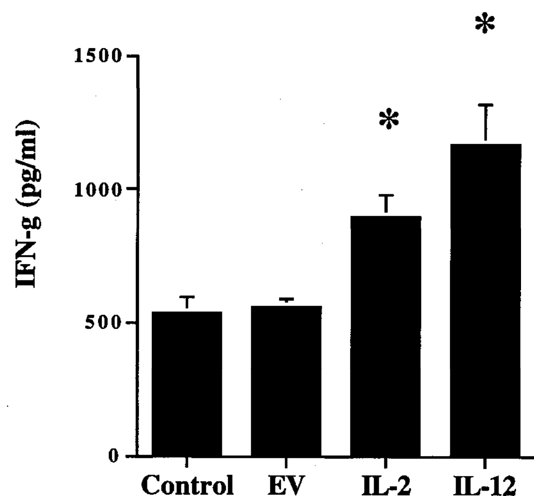

FIG. 8. Increased IFN-γ production by lung tissues after intravenous delivery of IL-2 or IL-12 genes. Lung tissues were collected from mice (four per treatment group) 48 hr after injection of lipid–DNA complexes containing either empty vector DNA, IL-2 DNA, or IL-12 DNA. The lung tissues were washed and minced with scissors, and then cultured in complete medium at 37°C for 18 hr. The supernatants were harvested and assayed for IFN-γ concentration by ELISA. The mean IFN-γ concentration ($\pm$SE) was plotted for each treatment group. ($^{*}p < 0.05$ compared with either control or empty vector-treated mice.)

activation by DNA–lipid complexes, plus immune enhancement induced by local cytokine production in the lungs.

Expression of a cytokine gene such as IL-2 or IL-12 in lung tissues after intravenous gene delivery may elicit local recruitment and activation of T cells and NK cells. In particular, binding and uptake of lipid–DNA complexes by the pulmonary vascular endothelium after intravenous injection may trigger vascular influx of inflammatory cells. Such a response is consistent with the histologic changes observed in tumor nodules of treated mice (Fig. 4). Inhibition of tumor growth after injection of lipid–DNA complexes may also have been mediated in part by inhibition of tumor angiogenesis. For example, IFN-γ is known to be a potent inhibitor of tumor angiogenesis (Coughlin *et al.*, 1998a,b). Both IL-2 and IL-12 can also trigger release of IFN-γ from NK cells and $CD8^+$ T cells (Fogler *et al.*, 1998; Khatri *et al.*, 1998; Whiteside *et al.*, 1998). Thus, the increased release of IFN-γ from the lungs of IL-2- or IL-12-treated mice may have reflected IFN-γ release from either of these two cell types (see Fig. 8).

The concentrations of IL-2 achieved in the lungs after systemic gene delivery were much lower than those that develop during systemic therapy with recombinant IL-2 (Rosenberg *et al.*, 1994; Fyfe *et al.*, 1996). Thus, local pulmonary expression of the IL-2 gene is unlikely to induce pulmonary vascular toxicity often associated with high-dose recombinant IL-2 therapy in humans. Furthermore, only low concentrations of IL-2 or IL-12 are required for effective augmentation of local immune responses. Preliminary studies of intravenous IL-2 gene delivery to dogs with advanced lung cancer metastases using DNA–lipid complexes indicate that such an approach is well tolerated in a large animal tumor model and can induce sustained tumor responses (R. E. Elmslie *et al.*, unpublished data, 1999). Thus, the systemic cytokine gene delivery approach described here may be broadly applicable to either treatment or prevention of cancer metastases to the lungs.

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