Intratumoral Injection of Interleukin-12 Plasmid DNA, Either Naked or in Complex with Cationic Lipid, Results in Similar Tumor Regression in a Murine Model

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Abstract
Effective eradication of established tumors and generation of a lasting systemic immune response is an important goal for cancer gene immunotherapy. The method of gene delivery may also be critical for the generation of an effective antitumor response. We compared the level of transgene expression and antitumor activity of two different interleukin (IL)-12 DNA preparations (naked DNA versus DNA lipid complex). Established murine adenocarcinoma (CT26) and renal cell carcinoma (Renca) tumors in BALB/c mice were treated by direct intratumoral injection of a nonviral plasmid DNA vector encoding the murine IL-12 (mIL-12) gene, either alone (naked) or in complexes with cationic lipid. Both treatments resulted in the same percentage (87%) of mice undergoing a complete tumor regression of the CT26 tumor. For the Renca tumor model, complete tumor regression was observed in 67 and 75% of animals treated with naked mIL-12 DNA and mIL-12 DNA plus lipid, respectively. Mice that were rendered tumor free for >50 days by mIL-12 gene therapy rejected a subsequent challenge of parental tumor cells but not of an unrelated, syngeneic tumor. The marked reduction of tumor growth in tumor-bearing mice treated with mIL-12 cDNA was associated with the augmentation of tumor-specific cytotoxic T cells, enhanced production of IFN-γ in spleen and lymph node cells, and increased splenomegaly and lymphadenopathy. The CD8⁺:CD4⁺ ratio in tumor-infiltrating lymphocytes was significantly increased in the tumor-bearing mice treated with mIL-12 DNA alone and mIL-12 cDNA plus lipid as compared with a control vector-treated group. These results indicate that direct intratumoral gene transfer with naked nonviral IL-12 DNA provides an effective and simple method for the treatment of murine tumors, suggesting an approach for clinical application.

Introduction
Effective eradication of established tumors and generation of a lasting systemic immune response with a simple gene delivery system are important goals for cancer gene immunotherapy. Cytokine genes are the most widely and extensively studied immunostimulatory agents in cancer gene therapy (1). In several comparative studies, IL-12 was the most effective cytokine gene that could induce eradication of experimental tumors, prevent the development of metastases, and elicit long-term antitumor immunity (2, 3). Depending on the tumor model, IL-12 can exert antitumor activities via T cells (4–8), NK cells (9–13), or NKT cells (14). Induction of cytokines, such as IFN-γ (4, 15–18) and IFN-inducible protein-10 (19), has also been implicated as a mechanism of antitumor activity of IL-12. In addition to its immunostimulatory properties, IL-12 induces antiangiogenic effects, thus inhibiting tumor formation and metastases (9, 20).

Local and systemic administration of IL-12 protein has been studied in various murine models (4, 5, 12, 21) and in Phase I/II human trials (22, 23). However, IL-12 protein therapy has been limited by dose-dependent toxicity (24, 25). Local and efficient expression of IL-12 and other cytokine genes in tumors represents an alternative immunotherapeutic approach that may avoid systemic toxicity of recombinant cytokines (6, 26, 27). Several methods to deliver the IL-12 gene, including particle-mediated gene transfer, have been studied in animal models (2, 5, 28, 29). Initial IL-12 gene therapy experiments were focused on retroviral vectors (30, 31). However, the use of retroviral vectors has disadvantages, such as low in vivo transduction efficiency, expression limited to dividing cells (32), and toxicity (33). Adenoviral recombinant vectors can infect nondividing cells, thus improving transfection efficiency, and can transduce in vivo cytokine genes, including IL-12, into tumors in animal models (12, 33–35). However, clinical applications with adenoviral vectors may be limited by possible systemic toxicities and by host immune response against viral proteins (34, 36). This antiadenoviral response may prevent repeated systemic administration of adenoviral vectors. High-dose application of adenovirus vectors for gene transfer has also been associated with severe clinical toxicity (37). In contrast, nonviral

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5 The abbreviations used are: IL, interleukin; mIL, murine IL; LN, lymph node; NK, natural killer; TIL, tumor infiltrating lymphocyte; CMV, cytomegalovirus; SI, stimulation index.
vectors, such as naked plasmid DNA and DNA complexed to cationic liposome systems, have thus far exhibited little immunogenicity and toxicity after repeated administrations (35, 38–43).

The method of gene delivery may also influence the generation of an effective antitumor response. Direct intratumoral DNA administration is reliable and reproducible and may limit the need for systemic cytokine administration (35, 38–40). Intratumoral injection of certain immunomodulatory genes can help elicit a host antitumor immune response (36, 43). Our group has shown that particle-mediated intratumoral treatment with a nonviral vector encoding mIL-12 cDNA resulted in 70% tumor regression of established CT26 liver and skin tumors (28). A complex of cationic liposome with DNA may result in higher intracellular uptake of the DNA, causing higher levels of transcription and higher levels of secreted cytokine. This may improve the antitumor efficiency of cytokine gene transfer (35, 39, 40, 43). To our knowledge, a functional comparative study of DNA complex with lipids and naked DNA alone has not been reported previously in a direct tumor treatment study. We report here the antitumor effects of direct intratumoral injection of the mIL-12 cDNA, either as naked DNA or in a liposome complex. We demonstrate that these two different DNA preparations can induce a similar therapeutic antitumor efficacy in the CT26 and Renca murine tumor models.

Materials and Methods

Tumor Cell Lines. CT26, an undifferentiated murine adenocarcinoma that was induced by rectal injection of N-nitroso-N-methylurethane in BALB/c mice (44), was kindly provided by Dr. Nicholas Restifo (Surgery Branch, National Cancer Institute, Bethesda, MD). This tumor and Renca, a spontaneously arising murine renal cell carcinoma (5), are syngeneic in BALB/c mice. Cell lines were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) cell culture medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal Eagle’s medium nonessential amino acids (BioWhittaker), and 10 μg/ml ciprofloxacin (Bayer, West Haven, CT). The RPMI 1640 culture medium with supplements was referred to as complete RPMI medium.

Animals. BALB/c female mice, 6–8 weeks of age, were obtained from Harlan Sprague Dawley Animal Facility (Indianapolis, IN) and Taconic Farms (Germantown, NY). Animals were housed and fed standard mouse chow and water ad libitum. All animal experiments were conducted in accordance with principles stated in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23; NIH, Bethesda, MD, 1985).

Plasmids. The mIL-12 DNA expression plasmid vector pNGVL3-mIL12 (28, 29) was −6.3 kb and constructed using a CMV early enhancer/promoter/intron-based plasmid with a kanamycin selection gene. The p35 and p40 subunits were separated by an internal ribosomal entry site and driven by a single CMV promoter (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI). pCMVLux, a control plasmid DNA vector (−5.6 kb) containing a luciferase gene with the CMV promoter, was constructed as described (2, 5).

Plasmid DNA was purified in the absence of ethidium bromide or penicillin derivatives by using a commercially available column chromatography method according to the manufacturer’s protocol (Qiagen, Chatsworth, CA).

Tumor Therapy in Vivo. For induction of tumor, the abdominal skin was shaved, and BALB/c mice were injected intradermally with 1.0 × 10^6 CT26 cells or Renca cells in 50 μl of PBS. Treatments began 7–10 days later, when tumor sizes ranged between 15 and 20 mm² in cross-section areas. Mice were given three treatments every other day. For intratumoral injections of naked DNA, DNA (10, 20, and 50 μg) was diluted in 100 μl of sterile PBS, left at room temperature for 15 minutes, and then injected into the tumor center via insulin syringes (28.5 gauge, 0.5-inch needles; Becton Dickinson, Franklin Lake, NJ). For intratumoral injections of liposome-DNA complex, the mIL-12 plasmid liposome DNA complex was made immediately prior to injection. The cationic lipid DMRIE/DOPE [(+/-)-N-(2-hydroxyethyl)-N,N-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide/dioleoylphosphatidylethanolamine] (Vical, Inc., San Diego, CA) was diluted with pyrogen-free sterile water to a concentration of 1 mg/ml total lipid containing 0.96 mg/ml DMRIE and 1.12 mg/ml DOPE and vortexed at top speed for 1 min at room temperature. The DNA/lipid complex was prepared by gently vortexing plasmid DNA (1 μg/μl) in sterile PBS with lipid to yield a DNA/lipid mass ratio of 5:1. The mixture solution was left at room temperature for 15 min. Sterile PBS was added to the liposome-DNA solution to a volume of 100 μl/dose and injected into the center of established tumors via insulin syringes.

Tumor growth was monitored two times/week by measuring two perpendicular tumor diameters with an electronic digital caliper. Mice were euthanized when the tumor area reached 100 mm².

Gene Expression in Situ. Gene-treated tumor sites were harvested at 24 h after the final treatment, homogenized in 0.5 ml of general extraction PBS containing 0.1% Triton X-100 and 1 mM Pefabloc (Boehringer Mannheim, Indianapolis, IN), and sonicated. Supernatant was removed after centrifugation of homogenized tissue solution and frozen at −20°C until ELISA assay. The expression of mIL-12 protein after gene treatment was measured by using a commercially available mIL-12 p70 ELISA kit with a sensitivity of 7.8 pg/ml (Biosource, Camarillo, CA).

Tumor Rechallenge. Treated mice exhibiting complete regression of CT26 tumor for 50 days were divided into two groups and rechallenged intradermally with 1 × 10^5 of either parental CT26 tumor cells or irrelevant syngeneic Renca tumor cells. Similarly, mice with IL-12-induced complete regression of Renca tumor for 50 days were divided into two groups and rechallenged with 1 × 10^5 of either parental Renca tumor cells or irrelevant syngeneic CT26 tumor cells. The tumor growth was monitored once a week, and mice were sacrificed when tumor reached 100 mm².

Cytotoxicity Assay. Spleens and axillary LNs were harvested from two mice/experimental group (pCMVLux control vector alone, naked or liposome mIL-12 plasmid DNA) 1 day after the last intratumoral gene treatment. The spleens and LNs were mechanically dissociated and passed through 70
μm filter to obtain a single cell suspension. RBCs were lysed by ACK lysis buffer (BioWhittaker) and washed three times with HBSS (BioWhittaker). Splenocytes and LN cells from each group were pooled and cocultured in vitro with irradiated (10,000 rads) CT26 tumor cells at an effector cell:stimulator ratio of 80:1 in 2 ml of complete RPMI 1640 supplemented with 50 μM 2-mercaptoethanol for 4 days at 37°C in 5% CO₂. After coculture, effector cells were collected from 24-well culture dishes and then used as effector cells in a 15-h chromium release assay against CT26 or unirradiated syngeneic Renca targets. Tumor target cells were labeled with 250 μCi of 51Cr for 2 h at 37°C, after which 1.5 × 10⁶ effector cells in 100 μl of complete RPMI 1640 were mixed with 5 × 10⁵ labeled target cells and added into 100-μl test wells in triplicate to produce an effector:target ratio of 30:1. Plates were centrifuged at 100 × g for 3 min and then incubated for 15 h at 37°C. Supernatants were harvested with the Skatron harvesting system (Skatron, Sterling, VA) and counted for 1 min in a gamma counter. The percentage of cytotoxicity mediated by CTLs was calculated as follows:

\[
\% \text{ of cytotoxicity} = \frac{\text{Exp. cpm} - \text{Spon. cpm}}{\text{Max. cpm} - \text{Spon. cpm}} \times 100\%
\]

where Exp. was the experimental number of counts from target cells incubated with the effectors, Spon. was the spontaneously released counts obtained from targets cultured in medium alone, and Max. was the maximum counts obtained from target cells completely lysed with a 2% cetrimide solution (Sigma Chemical Co.).

Cytokine Production in Vitro and in Vivo. Splenocytes and LN cells were isolated as described above for sensitization in the cytotoxicity assay. After coculture, cell culture supernatants were collected and frozen at −20°C for IFN-γ measurement by ELISA using commercially available reagents. The ELISA assays were prepared using the appropriate purified and bionylated rat antimouse antibody pair (PharMingen, San Diego, CA), streptavidin-horseradish peroxidase conjugate (Zymed, South San Francisco, CA), and tetramethylbenzidine substrate (Dako, Carpinteria, CA). The lower limit of detection was 78 pg/ml. All samples were assayed in duplicates.

For the in vivo time course cytokine production experiment, CT26 tumor-bearing mice were treated with intratumoral injection of pCMVLux control vector, naked or liposome mIL-12 plasmid DNA at 50 μg/treatment for three times. Mice were sacrificed, and spleens, axillary LN, and the tissue of treatment sites were harvested at 1, 3, 7, and 14 days after their last gene therapy. Treated sites were homogenized in general extraction PBS, and the homogenized solution was centrifuged. Supernatant was collected, and the expression of mIL-12 and IFN-γ protein was determined by specific ELISAs as described above.

Changes in Spleens and LNs after mIL-12 Gene Treatment. Spleens and LNs were obtained as described above. The weight of spleens and LNs was recorded, and the tissues were mechanically dissociated and passed through 70 μm mesh filters to obtain single cell suspensions. Cell numbers of each lymphoid organ were determined by hemacytometer. SI of tissue weight and cell number were calculated by the following formula: Tissue weight SI = mean tissue weight of a lymphoid organ obtained from mIL-12 treated tumor-bearing mice/mean tissue weight of a lymphoid organ obtained from PBS or lipid alone (control) treated tumor-bearing mice; Cell number SI = mean cell number in a lymphoid organ obtained from mIL-12-treated tumor-bearing mice/mean cell number in a lymphoid organ obtained from PBS or lipid alone (control) treated tumor-bearing mice.

Splenocytes and LN cells were isolated as described above. TILs were isolated using a modified, previously described procedure (45). Briefly, the tumor specimen was excised and minced in the presence of 1:10 volume enzyme mixture consisting of 200 units/ml collagenase I (Worthington, Freehold, NJ), 200 units/ml hyaluronidase (Sigma Chemical Co.), and 100 units/ml DNase (Dornase alfa; Genentech, Inc. South San Francisco, CA). The enzyme solution was prepared in RPMI 1640. minced tissue was transferred to a 50-ml trypsinizing flask containing 4-mm glass beads and 20 ml of enzyme solution. The mixture was incubated for 30 min at 37°C in a 5% CO₂ incubator with gentle stirring. Cells recovered were then washed twice in complete RPMI 1640 and then spun over Ficoll gradient (Lymphoprep; Life Technologies, Inc., Grand Island, NY) in 15-ml centrifuge tubes. Cells were harvested from the interface and washed with HBSS twice. Immune cells were prepared for the analysis of CD4-, CD8-, and NK-positive cells using a FACSscan flow cytometer (Becton Dickinson, Mountainview, CA). Cells (10⁶) were incubated with 1 μg of FITC-labeled anti-CD4 monoclonal antibody, phycoerythrin-labeled anti-CD8 monoclonal antibody, or FITC-labeled anti-pan-NK antibodies along with respective isotype control antibodies (all obtained from Pharmingen) on ice for 45 min. Cells then were washed and resuspended in 400 μl of PBS plus 2 μg of propidium iodide (Sigma Chemical Co.). Cytometric analysis was performed using the CellQuest analysis program (Becton Dickinson).

Statistical Analysis. Comparisons of the number of disease-free mice at 50 days between experimental groups were performed with Fisher’s exact test. The differences in the survival rate between experimental groups were analyzed with the Kaplan-Meier survival analysis and the log-rank test methods. All analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC). Findings were regarded as significant if the two-tailed P was ≤0.05. For other data analysis including cytotoxicity assays, level of cytokine production, SI, and phenotypic analysis, the significance of differences between the mean value of control groups and mean value of the test groups, as well as comparisons between the various treatment groups, was analyzed using Student’s t test. P < 0.05 was considered to be significant.

Results
Levels of Transgene Expression in Treated Tumor Site. To compare the efficiency of gene transfection, expression of IL-12 transgenic protein in the treated tumor site was evaluated by ELISA. mIL-12 protein was detected in all resected samples treated with naked or liposome DNA but not in
control groups (Fig. 1). There was no significant difference in the mIL-12 protein expression level between naked and liposome-DNA treated groups for both CT26 and Renca tumors. Levels of mIL-12 protein in CT26 treated with naked or liposome DNA ranged from 400 to 800 pg/tissue biopsy. Levels of mIL-12 protein in Renca treated with naked DNA ranged from 200 to 600 pg of tissue biopsy, whereas the levels of transgenic mIL-12 protein in Renca treated with liposome DNA ranged from 500 to 900 pg of tissue biopsy. The level of protein expression was thus quite similar when identical amounts of cDNA were introduced either with or without lipid.

**In Vivo Kinetic Study of Cytokine Production in Local Tumor Sites.** The expression of mIL-12 protein in the treated site of CT26 tumors remained relatively stable for 14 days after the last DNA injection. No significant difference was noted between naked and liposome DNA-treated tumors, although the levels of IL-12 expression were generally higher in the liposome DNA-treated groups (Fig. 2A).

The biological effect of IL-12 can be evaluated by induction of in vivo IFN-γ production in the treatment site. The various tissue extracts described above were also tested for IFN-γ levels by ELISA. Direct intratumoral injection of naked or liposome mIL-12 gene resulted in a marked increase (P < 0.001) of IFN-γ level compared with results from the pCMVLux-treated group when measured on days 1, 3, 7, and 14 after the last gene delivery (Fig. 2B). No difference in IFN-γ induction was noted when naked DNA and liposome DNA injections were compared.

**Antitumor Efficacy Is IL-12 cDNA Dose Dependent and Independent of Liposome Complex.** A dose-dependent antitumor response against CT26 adenocarcinoma was noted in both groups receiving either naked or lipid IL-12 cDNA. Intratumoral delivery of 50 μg of cDNA for each of three treatments resulted in tumor regression in 95% of animals (Fig. 3). The rate of tumor growth in groups treated with 50 μg of cDNA in naked or liposome forms was significantly lower (P < 0.001) than the rate of tumor growth in groups treated with 10 or 20 μg of cDNA, which, in turn, was significantly lower (P < 0.001) than the rate of tumor growth.
in the control groups (pCMVLux cDNA or lipid alone treated groups; Fig. 3). The survival rate of tumor-bearing mice when treated with 50 μg of DNA in naked or liposome forms was also significantly greater than that of the tumor-bearing mice treated with 10- or 20-μg DNA groups (P < 0.005). The latter was in turn significantly higher than survival of control groups (P < 0.001), such as pCMVLux cDNA or lipid alone (Fig. 4).

To validate that direct intratumoral injection of mIL-12 cDNA can confer antitumor effects in different types of tumors, a second intradermal tumor model of Renca (also syngeneic in BALB/c mice) was evaluated. Injections of naked or liposome-IL-12 plasmid cDNA at 50 μg/treatment into established Renca tumors, every other day for three treatments, were performed in a manner identical to the CT26 experiment. Tumor growth rate was significantly lower (P < 0.001) in IL-12 cDNA treated groups (naked or liposome DNA) than that in control vector (pCMVLux) treated groups (Fig. 5). The number of mice with complete tumor regression in cDNA-treated groups (naked or liposome DNA) was significantly higher than that seen in the control groups (P < 0.005; data not shown). No difference between liposome or naked IL-12 cDNA at the 50-μg dose was observed (Fig. 5).

**Tumor-specific Immune Response after mIL-12 cDNA Treatment.** Between 50 and 80 days after the primary tumor challenge with CT26 or Renca, all mice with complete tumor regression were rechallenged with either CT26 or Renca tumor cells. Results showed that 96% of mice with CT26 regression after mIL-12 gene therapy survived rechallenge with CT26, but only 23% of these mice survived when rechallenged with Renca (Table 1). Similarly, 100% of mice with Renca regression after mIL-12 gene therapy survived when rechallenged with Renca, but none (0%) of these mice survived rechallenge with CT26 tumor cells (Table 1). Thus, the IL-12 cDNA treatment induced tumor regression and resulted in tumor-specific immunological memory.

Table 1 Survival percentage of mice after tumor rechallenge

<table>
<thead>
<tr>
<th>First Tumor, Second Challenge</th>
<th>CT26 Regression No. Alive/Total</th>
<th>Renca Regression No. Alive/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT26</td>
<td>23/24 (96%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>Renca</td>
<td>5/22 (23%)</td>
<td>9/9 (100%)</td>
</tr>
</tbody>
</table>

**Induction of Splenomegaly and Lymphadenopathy by Direct Intratumoral Injection of mIL-12 Gene.** Direct intratumoral injection of naked or liposome IL-12 cDNA induced marked splenomegaly and lymphadenopathy. The tissue weight and the number of cells in both spleen and LN significantly increased (1.5–2-fold) in mice at 1, 3, and 7 days after the last mIL-12 gene therapy, compared with these same time points in mice treated with the pCMVLux control vector (P < 0.05; Fig. 6). The weight in the IL-12 cDNA-treated group returned to the level of the control group by day 14 (Fig. 6A). Similarly, the increased cell number of spleens and LNs noted between days 1 and 7 after the last mIL-12 gene therapy also decreased to the level of the control groups by day 14 (Fig. 6B). No statistical differences in increasing tissue weight and the number of cells in spleens and LNs were observed between naked and liposome IL-12 cDNA treatment groups (Fig. 6).

**Intratumoral Injection of mIL-12 Plasmid DNA Generates Tumor-specific CTL Response and Elevated IFN-γ Production by Splenocytes and LN Cells.** To compare the effect of IL-12 cDNA treatments on CTL activity of immune effector cells, both splenocytes and LN cells of CT26 tumor-bearing mice were evaluated. Cells were cocultured with irradiated CT26 tumor and used as effectors in 51Cr-release assays. Splenocytes of CT26 tumor-bearing mouse treated
with IL-12 cDNA were able to serve as more effective killers than the splenocytes of control vector-treated mice (38% versus 8% cytotoxicity). Comparable induction of CTLs was observed for groups treated with naked or liposome complex IL-12 cDNA. LN cells of CT26 tumor-bearing mice treated with mIL-12 cDNA were also superior to control LN cells in the CTL assay (48% versus 12% cytotoxicity; Fig. 7A). No difference in lysis of Renca tumor cells was detected between mIL-12-treated and control animals (Fig. 7B).

The IFN-γ levels of cocultured splenocytes or LN cell supernatants were compared. Enhanced secretion of IFN-γ by cocultured splenocytes was observed in animals that had been treated with naked DNA (21 ± 1.5 ng/10^6 cells/24 h) or liposome DNA (22 ± 2.5 ng/10^6 cells/24 h), compared with the splenocytes from mice treated with pCMVLux control vector (0.2 ± 0.01 ng/10^6 cells/24 h; P < 0.001). Supernatants from LN cell cocultures also showed marked difference in the IFN-γ levels (26 ± 2 and 24 ± 3 ng/10^6 cells/24 h for the supernatants of mice treated with naked or liposome DNA therapy, respectively, as compared with 0.1 ± 0.01 ng/10^6 cells/24 h for the LN cells of mice treated with pCMVLux control vector (P < 0.001; Fig. 7C). There was no statistically significant difference in the IFN-γ production in supernatants from naked mIL-12 cDNA or liposome mIL-12 cDNA for either spleen cells or LN cells.

Phenotypic Changes of Immune Effector Cells in Tumor Local Site. Phenotypic analysis of the spleen cells, axillary LN cells, and TILs from pCMVLux-, naked mIL-12 DNA-, or liposome mIL-12 DNA-treated CT26 tumor-bearing mice at 24 h after the last gene therapy was carried out by flow cytometry. Among the three treatment groups, there was no significant difference in the distribution of CD4+ T cells, CD8+ T cells, and NK cells in spleens and LNs of tumor-bearing mice treated with pCMVLux control vector alone, naked mIL-12 cDNA, or liposome mIL-12 cDNA (Table 2). As expected, a marked increase (P < 0.05) in the CD4+ : CD8+ ratio was induced in TILs at the tumor site by the injection of naked or liposome mIL-12 plasmid DNA (CD4+ : CD8+ ratio = 2.4 ± 0.3 and 3.0 ± 1.1 for naked or liposome DNA-treated tumor, respectively) compared with the pCMVLux DNA-treated control group (CD8+ : CD4+ ratio = 1.2 ± 0.6; Table 2). No significant changes have been observed in
the NK+ cell percentage in TILs among the various treatment groups (Table 2).

**Table 2** Percentage of CD4+, CD8+, and NK cells in spleens and LNs or CD8:CD4 ratio in TILs of CT26 tumor-bearing mice at 24 h after last gene therapy.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Spleens</th>
<th>LNs</th>
<th>TILs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+ (%)</td>
<td>CD8+ (%)</td>
<td>NK (%)</td>
</tr>
<tr>
<td>pCMVLux</td>
<td>27.75 ± 1.34</td>
<td>16.70 ± 2.26</td>
<td>4.95 ± 1.06</td>
</tr>
<tr>
<td>N-MiLL2 DNA</td>
<td>24.90 ± 0.57</td>
<td>15.40 ± 3.11</td>
<td>5.45 ± 1.63</td>
</tr>
<tr>
<td>L-miLL2 DNA</td>
<td>23.70 ± 1.70</td>
<td>16.00 ± 3.39</td>
<td>5.75 ± 1.20</td>
</tr>
</tbody>
</table>

*P < 0.05 when compared with pCMVLux control-treated group.

**Discussion**

IL-12 gene therapy may induce antitumor effects and potentially avoid the systemic toxicity associated with IL-12 protein immunotherapy (5, 27, 46–48). Prior studies have tested direct transfer of IL-12 cDNA into tumor, either using certain tumor cells engineered in vitro to release IL-12 (3, 30, 48) or using direct intratumoral injection of an adenovirus expressing IL-12 DNA (34) or a Canarypox virus expressing IL-12 vector (49). In addition, particle-mediated transfer of IL-12 cDNA was effectively used to treat murine tumors (2, 5, 28).

All of these methods could inhibit tumor growth and induce antitumor immunity. Direct intratumoral injection of IL-12 cDNA, either naked or in complex with a liposome formulation, may provide an alternative, simpler, and safer gene transfer method compared with localized or systemic viral gene vector therapy. Liposomes have been used previously as a delivery agent incorporating nonviral plasmid vectors encoding other cytokine genes, including IL-2 and IL-4, to successfully induce a complete regression of established tumor by direct intratumoral injection (6, 38). The combination of liposome-cDNA complex and IL-12 delivery directly into tumors combines the safety benefits of local delivery and avoidance of antigenetic viral constructs (22, 25). We have demonstrated in the present study that an even simpler delivery method (direct injection of naked nonviral plasmid IL-12 cDNA in an expression vector) can result in a similar gene expression and antitumor response as compared with liposome-cDNA complex.

Tumor-bearing mice demonstrated a dose-dependent response to plasmid DNA treatments. Three 50-μg direct intratumoral injections, using either nonviral IL-12 plasmid DNA alone or in complex with liposome, induced a complete tumor regression in the majority of treated mice. Doses of 10 or 20 μg of cDNA led to a marked decrease in tumor size, and 50% of the CT26 tumor-bearing mice was rendered tumor-free after gene treatments. The injection of IL-12 cDNA directly into tumor resulted in transgene expression for at least 2 weeks. There was no significant difference in the amounts of gene expression and secondary cytokine (IFN-γ) production induced by nonviral plasmid cDNA alone versus cDNA plus lipid in CT26 tumor-bearing mice. These results are consistent with that reported by Nomura et al. (35), who showed that although cationic liposome could enhance the retention of injected DNA in the tumor site, the level of gene expression was similar between naked DNA and its cationic liposome after direct intratumoral injection. The specificity of the IL-12 DNA-mediated anti-CT26 and anti-Renca tumor responses was demonstrated in reciprocal experiments by rechallenge with either CT26 or Renca tumor (Table 1). This finding is similar to previous reports of induction of tumor-specific immunological memory after IL-12 protein or gene therapy (4, 5, 34).

Tumor regression by IL-12 gene therapy is dependent on the presence of T cells, IFN-γ, and IFN-inducible protein-10 (4–8, 15–19). NK cells and NKT cells may also play an important role in IL-12 gene-mediated antitumor responses (9–14). IL-12 also has been shown to confer antiangiogenic activities that can account for some of the antitumor effects (9, 20). Spleen cells and LN cells from CT26 tumor-bearing mice that were treated with either naked IL-12 DNA or in complex with lipid, in contrast to spleen cells and LN cells from control vector-treated mice, exhibited CTL activity upon stimulation with irradiated CT26 tumor cells in vitro. The same effector cells did not lyse the syngeneic Renca tumor cells when they were included in these assays. These results demonstrate that tumor-bearing mice, which were treated with direct intratumoral injection of nonviral IL-12 gene, had developed tumor-specific immunological responses. In our previous study using the nonviral IL-12 plasmid DNA to treat CT26 liver and skin tumors, we found in T-cell subset depletion experiments that the IL-12-mediated tumor regression was dependent on CD8+ cells (28). In this report, we demonstrated that no detectable change in the percent of CD4+, CD8+ T cells or NK cells was found in the spleen or draining LNs, despite an overall increase in the size and number of lymphocytes found in both spleen and LNs. However, within the population of TIL cells, the CD8+:CD4+ ratio of tumor-bearing mice treated with IL-12 plasmid DNA was higher compared with control vector-treated mice. These results are consistent with our previous observation, indicating that CD8+ T cells play an important role in IL-12-mediated tumor regression and suggest that the local interaction of tumor with effector cells is critical for initiating tumor regression.

The induction of IFN-γ production by activated T and NK cells is a hallmark of IL-12-stimulated bioactivity in vitro and in vivo (4, 15–18). We have demonstrated that both spleen cells and LN cells from tumor-bearing mice treated with IL-12 plasmid DNA produced significantly higher amounts of IFN-γ as compared with the cells from tumor-bearing mice treated...
with control vector alone. These results suggest that these cells had been primed and activated in vivo after exposure to IL-12. Furthermore, we have demonstrated that the levels of intratumoral IFN-γ can last 14 days after the last IL-12 gene treatment. These results are consistent with previous findings demonstrating that the production of IFN-γ increases dramatically in tumors and tumor-draining LNs after intratumoral injection of adenoviral vectors expressing IL-12 genes (34). Other investigators have shown that the expression of IFN-γ mRNA increases significantly in tumors and spleens after systemic IL-12 treatment (18). The association of high-level production of IFN-γ by tumor-draining LN cells correlated with tumor rejection in IL-12-dependent models of tumor rejection (29, 44). Our data provide additional evidence that IL-12 gene delivery directly into tumors can alter the local cytokine environment by the induction of IFN-γ expression, suggesting that IL-12-mediated tumor regression may partially depend on IFN-γ (13, 29).

The results of this study also demonstrate that CT26 tumor-bearing mice treated with IL-12 nonviral plasmid DNA developed splenomegaly and lymphadenopathy in comparison to the mice treated with control vector. This splenomegaly and LN enlargement is consistent with observations made using IL-12 protein therapy (49, 50). These results, together with our in vitro studies (Fig. 7), suggest that IL-12 gene therapy induces accumulation of antitumor effector cells in LN and spleen. These may be necessary and important for induction of tumor regression and immunological memory responses.

In summary, our results demonstrate that multiple intratumoral injections of nonviral plasmid DNA can effectively eradicate established tumors in our model systems. This tumor rejection is associated with IFN-γ production at the treatment site, in tumor draining LNs and in spleen. CTL activities are generated by the induction of mlr-12 cDNA treatment. No differences in transgene expression levels, IFN-γ production, CTL generation, or immunological memory were noted when plasmid cDNA alone or plasmid cDNA complex to cationic lipid were injected into tumor. Furthermore, in vivo antitumor efficacy, measured as suppression of tumor growth or increase in survival of tumor-bearing animals, was similar for animals treated with these DNA doses of naked and lipid complex IL-12 cDNA. These comparative studies were shown to be reproducible in both CT26 and Renca tumor models. Obviously, the conclusion that the efficacy of naked DNA is similar to that of a cationic lipid is only relevant to the specific lipid used and at a specific ratio of DNA:lipid. Nevertheless, although these results might not necessarily be reproducible with other lipids or tumor models, the fact that naked DNA had comparable efficacy to lipid complex DNA in all tested comparisons, as shown here in two distinct tumor models, suggests that this simple method of DNA delivery may be applicable to the treatment of human cancers.

References


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Intratumoral Injection of Interleukin-12 Plasmid DNA, Either Naked or in Complex with Cationic Lipid, Results in Similar Tumor Regression in a Murine Model

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