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In Vivo Antitumor Activity of Interleukin 21 Mediated by Natural Killer Cells

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ABSTRACT

Immunotherapy with high-dose interleukin (IL) 2 has been shown to successfully treat tumors in animal models and cause dramatic tumor regressions in some patients with metastatic melanoma, renal cell carcinoma, and non-Hodgkin's lymphoma. However, toxicity associated with IL-2 administration has compromised its widespread use in the clinic. IL-21 is a more recently discovered cytokine produced by activated CD4$^+$ T cells that shares significant sequence homology to IL-2, IL-4, and IL-15. Because IL-21 and IL-2 and their receptors share significant sequence similarities and both cytokines can stimulate T and natural killer (NK) cells, we sought to study whether IL-21, like IL-2, exhibits antitumor effects *in vivo*. In this study, we treated established s.c. tumor in mice by systemically administering plasmid DNA encoding murine *IL-21* using a hydrodynamics-based gene delivery technique. Administration of IL-21 plasmid DNA resulted in high levels of circulating IL-21 *in vivo*. Treatment of tumor-bearing mice with IL-21 plasmid DNA significantly inhibited the growth of B16 melanoma and MCA205 fibrosarcoma in a dose-dependent manner without significant toxicity and increased the survival rate, compared with mice treated with control plasmid DNA. *In vivo* depletion of either CD4$^+$ or CD8$^+$ T cells did not affect IL-21-mediated antitumor activity. However, depletion of NK cells completely abolished IL-21-induced tumor inhibition. Consistent with this, the antitumor activity of IL-21 seemed to be mediated through enhanced cytolytic activity of NK cells. Our study suggests that IL-21 has significant antitumor activity and may have therapeutic potentials as an antitumor agent in the clinic.

INTRODUCTION

The administration of high-dose recombinant interleukin (IL) 2 has been shown to induce tumor regression in mouse tumor models (1) and has been successfully used to treat patients with metastatic melanoma, renal cell carcinoma, and non-Hodgkin's lymphoma (2–4). The effect of IL-2 on cancers is presumably derived from its ability to expand and activate lymphocytes with antitumor activity *in vivo*. *In vitro*, IL-2 can stimulate natural killer (NK) cell expansion and T-cell growth after activation by specific antigens. However, dose-limiting toxicities associated with IL-2 have compromised its clinical use, and other cytokines with an improved therapeutic index are needed.

IL-21 is produced by activated CD4$^+$ T cells and shares significant sequence homology to IL-2, IL-4, and IL-15 (5). IL-21 has potent effects on all classes of lymphocytes (B, T, and NK cells). It acts synergistically on T cells with a proliferative signal provided by anti-CD3 antibodies and promotes expansion of mature B cells in response to stimulation through CD40. In addition, IL-21, in synergy with Flt3 ligand and IL-15, promotes expansion and differentiation of NK cells from bone marrow progenitors *in vitro* and enhances lytic effector function against target cells in lysis assays (5, 6). IL-2 and IL-21 and their corresponding receptors share significant homology (5, 7). Both cytokines mediate T and NK cell responses (8), and their receptors share the γc chain. Hence, we sought to determine whether IL-21, like IL-2, exhibits antitumor activity *in vivo*.

To obviate the need to produce large amounts of IL-21 protein, we used a hydrodynamics-based gene delivery technique that involves the rapid i.v. injection of plasmid DNA encoding a transgene in a large volume of solution (9, 10). This method has been demonstrated to result in a prolonged expression of large amounts of circulating protein, primarily because of expression in hepatocytes (9, 10), and can be used efficiently to study the biological functions of potential therapeutic gene products *in vivo* without producing large amounts of proteins (11).

In the present study, we systemically administered plasmid DNA encoding murine (m) *IL-21* (mIL-21) into mice to treat established s.c. tumors and found that IL-21 significantly inhibited tumor growth *in vivo* without obvious toxicity and prolonged survival of tumor-bearing mice. This antitumor activity seemed to be mediated through the enhanced cytolytic activity of NK cells.

MATERIALS AND METHODS

Cell Lines and Reagents. B16 melanoma and MCA205 fibrosarcoma tumor lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, L-glutamine, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (all from Invitrogen/Life Technologies, Inc., Rockville, MD). Antimouse monoclonal antibodies against CD4 (GK1.5) and CD8 (2.43) were obtained from the National Cancer Institute Biological Resources Branch Preclinical Repository (Frederick, MD). Anti-asialo GM1 antibody against mouse NK cells was purchased from Wako Pure Chemical Industries (Osaka, Japan). Recombinant mIL-21 was purchased from R&D Systems (Minneapolis, MN). Antibodies used for fluorescence-activated cell sorting analysis were purchased from BD/PharMingen (San Diego, CA).

Cloning of mIL-21. Freshly isolated murine splenocytes from C57BL/6 mice were activated with 5 ng/ml phorbol 12-myristate 13-acetate and 250 μg/ml ionomycin for 24 h. Total RNA was extracted using TRIZOL (Invitrogen/Life Technologies, Inc.). Reverse transcription (RT)-PCR was performed to amplify the first strand of cDNA by random primers using ThermoScript RT-PCR System (Invitrogen/Life Technologies, Inc.). The full-length mIL-21 cDNA fragment was PCR amplified using PCR SuperMix High Fidelity (Invitrogen/Life Technologies, Inc.). The PCR primer sequences are 5′-CCACCGGCGGGTGGCATGGAGAGGACCCTTGTC-3′ and 5′-GCTAGCCTAGGAGAGATGCTGATGAAT-3′, which contain *Sgr*AI and *Nhe*I restriction enzyme sites, respectively. The full-length mIL-21 cDNA fragment was digested and cloned into the pORF-mcs vector under the control of an elongation factor-1α/human T-cell leukemia virus hybrid promoter (InvivoGen, San Diego, CA) and was designated as pORF/mIL-21. The sequence of PCR-amplified mIL-21 was confirmed as correct by sequence analysis. To exclude endotoxin contamination, large preparation of pORF/mIL-21 and the control pORF plasmid DNA was purified using the EndoFree Plasmid Mega kit (Qiagen, Valencia, CA).

Gene Delivery. Injection of plasmid DNA encoding mIL-21 or control vector pORF-mcs was performed using the hydrodynamics-based gene delivery technique (9, 10). Briefly, 8–10-week-old mice received i.v. injections of 2 ml of saline containing various amounts of plasmid DNA in 5–7 s using a 25-gauge needle. The volume of solution injected was based on the age and weight of mice and did not exceed 10% of body weight. Mice tolerated this treatment regimen well without obvious side effects observed after injection.

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The maximum tolerable DNA doses for mIL-2, mIL-4, and mIL-10 is 1 μg/mouse and 10 μg/mouse for murine tumor necrosis factor (TNF)-α and mIL-12, as determined by animal death after injection.

Tumor Inhibition Study. On day 0, 8–10-week-old C57BL/6 mice (National Cancer Institute) received s.c. inoculations of 5×10^5 B16 melanoma or MCA205 fibrosarcoma tumor cells. On day 5, tumor-bearing mice received i.v. injections of plasmid DNA dissolved in 2 ml of saline prewarmed to room temperature. Seven days later, the DNA injection was repeated. Mice were ear-tagged and randomized, and the tumor growth rate was determined by blindly measuring the perpendicular diameters of tumors two or three times per week using digital calipers. The tumor sizes were calculated by multiplying the length and width of each tumor. The mouse survival rate was also recorded.

***In Vivo* Cell Depletion Study.** *In vivo* CD4 and CD8 depletion was performed as described previously using antimouse CD4 (GK1.5) and CD8 (2.43) antibodies (12). Briefly, 2 and 4 days after tumor inoculation, tumor-bearing mice received i.v. injections of 200 μg/mouse of either anti-CD4 or -CD8 antibodies. The antibody injection was repeated i.p. every 6 or 7 days thereafter during the experiment to maintain the depletion of CD4 and CD8 cells. mIL-21 plasmid injection was performed on days 5 and 12. CD4 and CD8 knockout mice (The Jackson Laboratory, Bar Harbor, ME) were also used for similar studies. Additional mice were included for each depletion study to verify the depletion of CD4 and CD8 cells by fluorescence-activated cell sorting analysis. For *in vivo* NK cell depletion, anti-asialo GM1 antibody was used according to the manufacturer's instructions. Briefly, anti-NK antibody was injected i.v. into tumor-bearing mice at 2 and 4 days after tumor inoculation and then injected i.p. every 6 days thereafter throughout the experiment to maintain the depletion. Tumor treatment was started on day 5 and repeated 7 days later.

mIL-21 ELISA. An ELISA system was used to detect mIL-21 expression in mouse serum. Briefly, monoclonal antibodies against mIL-21 as a capture antibody were coated overnight onto a 96-well plate at 4°C. Serial dilutions of serum samples were added to the coated plate the next day and incubated at 4°C overnight. A biotin-labeled rat antimouse IL-21 polyclonal antibody was used as a detection antibody using standard methods (IL-21 antibodies were from R&D Systems).

Multiple Cytokine Immunoassay. C57BL/6 mice received i.v. injections of 20 μg of either pORF or pORF/mIL-21 plasmid DNA or saline alone. Positive control mice were injected with 1 μg of mIL-2, mIL-4, mIL-10, and mIL-12 plasmid DNA, respectively. Mice were sacrificed at 16 h or 4 or 8 days after injection, and serum levels of multiple cytokines were determined by an ELISA-based SearchLight murine cytokine array technology (Pierce/Endogen, Woburn, MA).

***In Vitro* Tumor Inhibition Assay.** The growth inhibition of tumor cells *in vitro* was determined by a 72-h 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium assay using the CellTiter 96Aqueous One Solution Assay kit according to the manufacturer's instructions (Promega, Madison, WI). Briefly, 1×10^5 murine tumor cells (including MCA205, B16, 24JK, and MC38) were plated in 24-well plates in 1 ml of RPMI complete medium in combination with various amounts of recombinant mIL-21 protein. After 3 days, 100 μl of culture medium from each well were collected and incubated with 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium reagent at 37°C for 2 h. Absorbance at 490 nm was then used to determine the relative cell growth between groups.

Fluorescence-activated Cell Sorting Analysis of Apoptotic Cells. Apoptosis was assessed by fluorescence-activated cell sorting staining of splenocytes using an Annexin V Apoptosis Detection kit from BD/PharMingen according to the manufacturer's instructions.

Cytotoxicity Assay. The cytolytic activity of NK cells was determined by a standard ^{51}Cr-release assay. Briefly, the effector NK cells were enriched from mouse spleens, 4 days after plasmid DNA injection, by DX5 MicroBeads (Miltenyi Biotec, Auburn, CA) using an AutoMACS separation system according to the manufacturer's instructions. The resulting NK1.1$^+$/CD3$^-$ cells were enriched by 10–15-fold, from 3.5 to 40% for NK cells from pORF-treated mice and from 3 to 25% for NK cells from mIL-21-treated mice. The enriched effector cells were incubated with ^{51}Cr-labeled B16 or YAC-1 target cells at different E:T ratios at 37°C for 4 h, and target cell lysis was calculated.

Statistics. The statistical analyses to compare tumor growth rate and mouse survival rate between treatment and control groups were determined by ANOVA-repeated measures test and Wilcoxon's rank-sum test using the StatView program (Abacus Concepts, Berkeley, CA). The statistical analyses to compare tumor sizes and cell numbers between treatment and control groups were determined by the nonparametric Kruskal-Wallis test using the StatView program.

RESULTS

Administration of mIL-21 Results in High Levels of Expression *in Vivo*. To study the *in vivo* effects of cytokines, we used a hydrodynamics-based gene delivery technique (9, 10). This method allows the prolonged production of large amounts of protein by hepatocytes after injection of plasmid DNA and is highly dependent on the volume and speed of injection. To determine the optimal promoter for our studies, we first compared the *in vivo* expression levels of a reporter gene, the chemokine *GRO-α*, in constructs with different promoters including Moloney murine leukemia virus long terminal repeat, cytomegalovirus, and an elongation factor-1α/ human T-cell leukemia virus hybrid promoter. We found that the elongation factor-1α/ human T-cell leukemia virus hybrid promoter generated the highest expression of the transgene *in vivo* after i.v. administration of plasmid DNA (data not shown). This vector (pORF-mcs) was subsequently used for the mIL-21 *in vivo* antitumor studies.

A full-length *mIL-21* gene including a signal sequence was amplified by RT-PCR from activated murine splenocytes and subsequently ligated into pORF-mcs. We then determined the time course of mIL-21 expression in mouse serum after direct injection of pORF/mIL-21 plasmid DNA into the tail vein. As shown in Fig. 1*A*, one day after a single dose of 20 μg of pORF/mIL-21 plasmid, a high level of mIL-21 was detected in mouse serum (6107 ± 2319 pg/ml) by a sandwich double antibody ELISA. Serum levels of mIL-21 decreased over time but were still as high as 278 ± 279 pg/ml on day 5 and returned to baseline on day 8. No detectable mIL-21 was seen in sera from naïve mice or mice that received injections of the same amount of control plasmid DNA.

For comparison, mice also received injections of several other cytokine DNAs, including mIL-2, mIL-4, mIL-12, and mTNF-α, all of which were constructed in the same pORF vector under the control of the same promoter, to test serum levels of cytokine proteins. As shown in Fig. 1*B*, IL-4, IL-12, and TNF-α, when injected at doses ranging from 1 to 10 μg/mouse, were expressed at levels similar to IL-21 during the 7-day period, whereas IL-2 was expressed at a much higher level even at a dose as low as 1 μg/mouse. Because the methods to determine the serum level of IL-21 and other cytokines differed, exact comparisons are not possible, although, except for IL-2, the pattern and extent of expression for each cytokine were comparable.

Importantly, we found no obvious toxicity caused by the *in vivo* expression of mIL-21 plasmid DNA at concentrations up to 100 μg/mouse. Extensive pathological examination and comparison of the mice that received injections of either mIL-21 or pORF plasmid DNA showed no evidence of major toxicities such as weight loss and capillary leaking, and so on, that have been associated with overexpression of cytokines *in vivo* (data not shown). In separate studies, we have found that injection of mIL-2, mIL4, or mIL-10 DNA at a dose of 2 μg/mouse resulted in severe toxicity, with all mice ($n = 6$) in each group dying within 5–10 days after injection, whereas three of six mice died after receiving an injection of 20 μg of mTNF-α.

mIL-21 Alters Splenocyte Subpopulations. To determine the effect of IL-21 expression on immune cell populations *in vivo*, flow cytometric analysis of mouse splenocytes was performed after plasmid administration. As shown in Table 1, 7 days after a single dose of 20 μg of mIL-21 plasmid, the percentage of CD3$^+$ and CD8$^+$ T cells

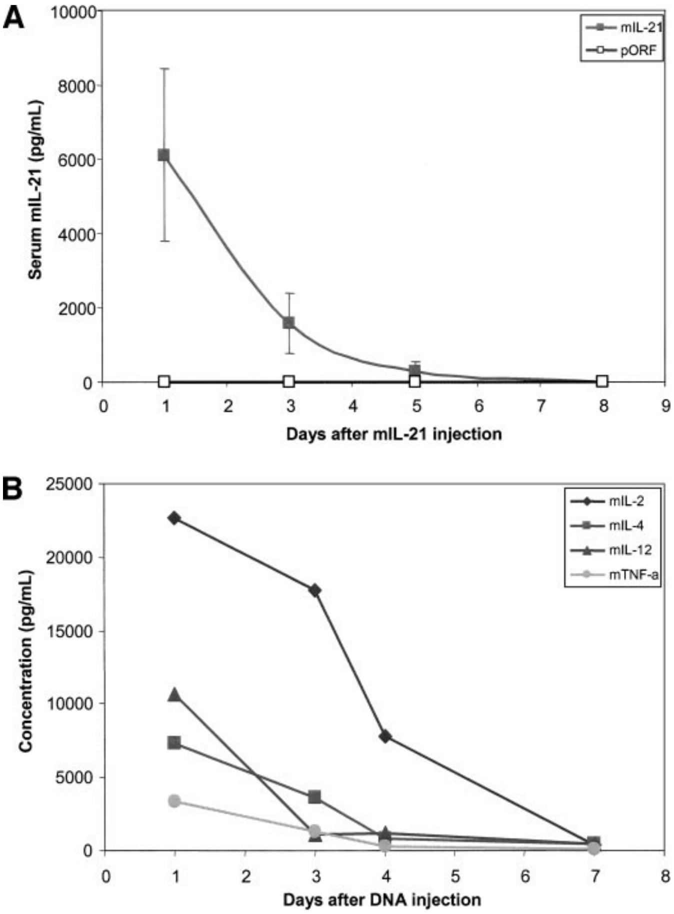

Fig. 1. Expression of mIL-21 and cytokines in mouse serum after DNA injection. *A*, C57BL/6 mice received i.v. injections of 20 μg of either mIL-21 or pORF control plasmid DNA in 2 ml of saline on day 0, as described in "Materials and Methods." On days 1, 3, 5, and 8, mice were sacrificed and serum samples were collected for mIL-21 ELISA analysis. Data are from one of two similar experiments. Each point represents data from three mice. *Bars*, SE. *B*, as in *A*, mice received injections of different cytokine DNA, as described in "Materials and Methods." On days 1, 3, 5, and 7, mice were sacrificed and serum samples were collected for cytokine analysis using an ELISA-based SearchLight murine cytokine array method. Each point represents the mean of three individual mice.

Table 1 *Effect of mIL-21 on immune cells in mouse spleens*

C57BL/6 mice received i.v. injections of 20 μg of either pORF or mIL-21 DNA plasmid. Seven days later, mouse spleen cells were harvested and total cell numbers were counted. Cell suspensions were stained with fluorochrome-conjugated-specific antibodies and subjected to fluorescence-activated cell sorting analysis. Three mice were in each group. Data represent one of five independent experiments with similar results.

	% of positive cells		Total no. of cells ($\times 10^6$)	
	pORF	mIL-21	pORF	mIL-21
CD3	39.3 ± 5.3	51.3 ± 2.2[a]	22.6 ± 1.8	45.3 ± 8.3[a]
CD4	26.0 ± 1.5	28.1 ± 2.4	15.0 ± 1.2	24.9 ± 5.6[a]
CD8	19.8 ± 4.0	26.8 ± 0.9[a]	11.3 ± 1.6	23.6 ± 3.8[a]
$NK1.1^+/CD3^-$	3.0 ± 0.3	0.7 ± 0.1[a]	1.7 ± 0.4	0.7 ± 0.2[a]
$DX5^+/CD3^-$	3.6 ± 0.3	1.3 ± 0.2[a]	2.1 ± 0.5	1.1 ± 0.3[a]
B220	63.2 ± 5.2	53.2 ± 2.7[a]	36.8 ± 7.7	46.6 ± 6.4
CD11b	14.0 ± 0.7	31.4 ± 0.6[a]	8.1 ± 1.3	27.6 ± 4.1[a]
CD11c	7.8 ± 0.4	9.6 ± 1.2	4.5 ± 0.8	8.4 ± 0.8[a]
Gr-1	11.4 ± 1.0	18.5 ± 1.6[a]	6.6 ± 0.9	16.2 ± 1.3[a]

[a] Significant differences between pORF and mIL-21 groups ($P < 0.05$).

in the spleen significantly increased in mIL-21-treated groups compared with the pORF control groups (51.3 ± 2.2 *versus* 39.3 ± 5.3% and 26.8 ± 0.9 *versus* 19.8 ± 4.1%, respectively; $P = 0.0219$ and 0.0418, respectively). Moreover, the percentage of cells in the myelomonocytic lineage as defined by CD11b and Gr-1 staining in the spleen was also significantly increased after mIL-21 administration (14.0 ± 0.7 *versus* 31.4 ± 0.6% and 11.4 ± 1.0 *versus* 18.5 ± 1.6%, respectively; $P < 0.0001$ and $P = 0.0027$, respectively). However, the percentage of mouse NK cells, as defined by $NK1.1^+/CD3^-$ or $DX5^+/CD3^-$ subpopulations, from spleen was significantly decreased in the mIL-21-treated group compared with the pORF control group (3.0 ± 0.3 *versus* 0.7 ± 0.1% and 3.6 ± 0.3 *versus* 1.3 ± 0.2%, respectively; $P = 0.0002$ and 0.0001, respectively). Similar changes in the phenotype of immune cells comparable with those seen in splenocytes were observed in mouse peripheral blood (data not shown). Because the spleen increased in size, weight, and total cell number after mIL-21 plasmid administration (data not shown), the increase in the absolute number of T-cell and myelomonocytic cell subpopulations was even more profound than the percentage increase in these populations in mIL-21-treated mice compared with control mice (Table 1). These observations suggested that the functional expression of mIL-21 *in vivo* after DNA injection had multiple biological effects on murine immune cells.

mIL-21 Significantly Inhibits Tumor Growth *in Vivo* and Prolongs Survival of Tumor-bearing Mice. To study whether systemic expression of IL-21 can inhibit tumor growth *in vivo*, mIL-21 plasmid was injected 5 days after s.c. tumor implantation and repeated 7 days later, based on the mIL-21 expression time course. We first determined the response of a fibrosarcoma tumor line, MCA205, to increasing doses of mIL-21. As shown in Fig. 2, all doses of plasmid DNA, except the lowest dose of 5 μg, significantly inhibited 5-day s.c. MCA205 tumor growth in a dose-dependent fashion ($P = 0.347$, 0.009, 0.009, and 0.009 for 5, 10, 15, and 20 μg, respectively), with a maximum inhibition of 55% at a 20-μg dose level of mIL-21 plasmid (183 ± 25 *versus* 410 ± 37 mm^2; $P = 0.0039$) on day 31. Administration of the same amount of control pORF DNA had no effect on tumor growth. Importantly, no obvious toxic effects were observed in treatment mice exposed to this high level of mIL-21. In comparison, tumor-bearing mice that were injected with 1 μg of mIL-2 DNA, which is the maximum tolerable IL-2 plasmid dose in mice, exhibited no antitumor effect ($P = 0.602$; Fig. 2).

To determine whether the treatment effect of IL-21 also inhibited the growth of other types of tumors, we treated B16 melanoma, a weakly immunogenic and more aggressive tumor, with mIL-21 in a 5-day s.c. model. As shown in Fig. 3*A*, mIL-21 treatment also significantly inhibited B16 melanoma growth *in vivo* ($P < 0.0001$). Of the

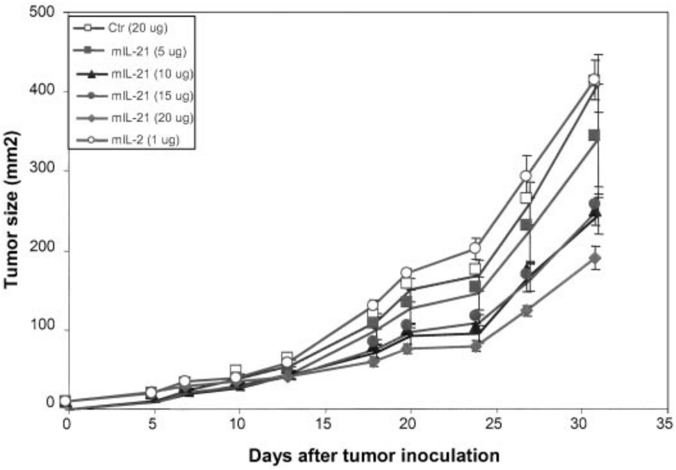

Fig. 2. Dose response of mIL-21 in the treatment of MCA205 tumor *in vivo*. C57BL/6 mice received s.c. inoculations of 5×10^5 MCA205 tumor cells on day 0. Five days later, tumor-bearing mice received i.v. injections of various doses of mIL-21 plasmid DNA ranging from 5 to 20 μg/mouse. Control mice received injections of either 20 μg of pORF or 1 μg of mIL-2 plasmid DNA. Injections were repeated 7 days later. Each group consisted of five mice. *Bars*, SE.

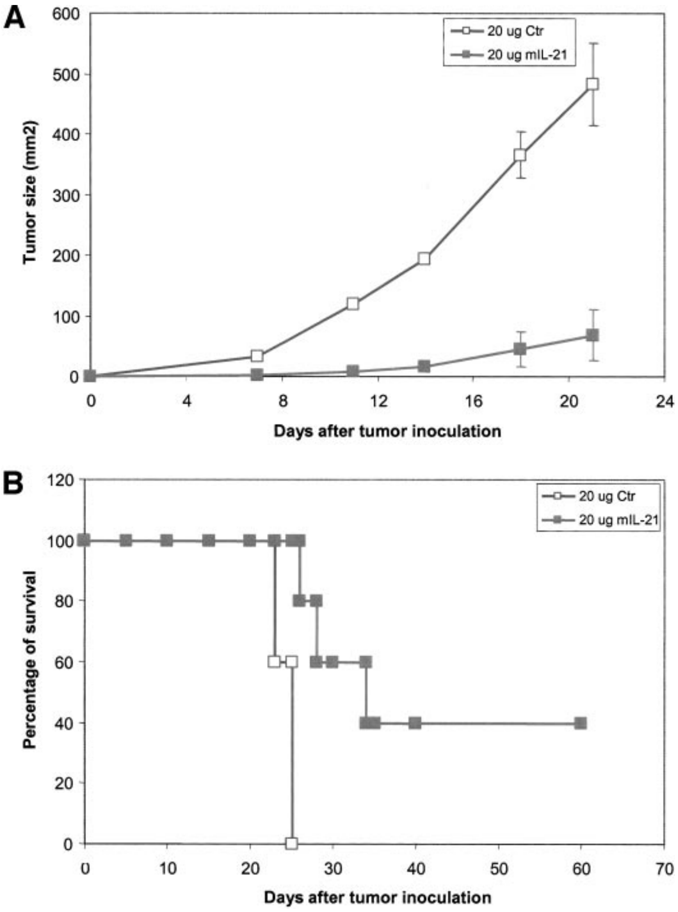

Fig. 3. Injection of mIL-21 plasmid DNA significantly inhibits B16 tumor growth *in vivo* and increases the survival rate of tumor-bearing mice. *A*, inhibition of B16 tumor growth *in vivo*. *B*, survival of B16 tumor-bearing mice after mIL-21 treatment. C57BL/6 mice received s.c. inoculations of 5×10^5 B16 tumor on day 0 and were treated with either 20 μg of mIL-21 DNA or pORF control DNA on days 5 and 12. Tumor growth and mouse survival rate were recorded. Data represent one of three experiments with similar results. Each group consisted of five mice. Differences between control and treatment groups in *A* and *B* are highly significant ($P = 0.0001$ and $P = 0.0031$, respectively).

five mice treated with mIL-21 in this experiment, two had a complete regression of tumor, and the other three had residual tumors much smaller than the tumors found in the five control mice. The survival of mIL-21-treated B16-bearing mice was also significantly longer than that of control mice (Fig. 3*B*; $P = 0.0031$). By day 25 after tumor inoculation, all mice in the control group had died, whereas 80% of mice in the treatment group were still alive. This experiment was repeated three times with similar results. Similar antitumor effects of IL-21 on another colon carcinoma tumor line, MC38, were also observed (data not shown).

mIL-21 Does Not Directly Inhibit Tumor Growth *in Vitro*. To determine whether recombinant IL-21 protein exhibits a direct inhibitory effect on the tumor cells used in our *in vivo* antitumor experiments, we performed a 72-h 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium tumor growth inhibition assay. As shown in Fig. 4, in the range of 20–100 ng/ml, recombinant mIL-21 did not directly inhibit the growth of the four tumor lines tested, including MCA205 and B16, consistent with the lack of expression of IL-21 receptor on these cell lines, as evaluated by RT-PCR (data not shown). Consistent with this finding, flow cytometric analysis of tumor cells for annexin V indicated no increase in tumor apoptosis after IL-21 treatment (data not shown). These results indicate that IL-21 does not have a direct inhibitory or cytotoxic effect on the tumor cells used in these studies and other mechanisms, such as stimulation of immune cells, must account for the observed *in vivo* antitumor activity.

IL-21 Does Not Induce Secretion of Other Cytokines. IL-2 administration is known to up-regulate multiple cytokines, and hydrodynamics-based gene delivery may itself up-regulate IL-12 and TNF-α (13). To determine whether IL-21 induced the secondary secretion of other cytokines that may have contributed to the antitumor response, we tested serum samples for a number of cytokines including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, and TNF-α using a multiple cytokine immunoassay. Sixteen hours and 4 and 8 days after a single injection of either the mIL-21 or pORF plasmid (20 μg each) or saline, none of the cytokines tested (including IL-2, IL-12, IFN-γ and TNF-α), which are known to have antitumor effects, was consistently elevated. Modest elevations in IL-6, IL-10, and IFN-γ in mIL-21-treated mice, which were attributable to higher levels in only one of the three mice tested in that group, were observed only on day 8, as seen by the high SD for these values. Serum samples from mice that received injections of mIL-2, mIL-4, mIL-10, and mIL-12 plasmid DNA served as positive controls and all showed high levels of the corresponding cytokines (Table 2). This result suggests that the antitumor effects of IL-21 are not mediated by these cytokines.

NK Cells Are Involved in the Antitumor Activity Induced by mIL-21. Because the injection of mIL-21 resulted in an expansion of $CD4^+$ and $CD8^+$ lymphocytes in the spleen (Table 1) and peripheral blood, we depleted $CD4^+$ or $CD8^+$ T cells *in vivo* using specific monoclonal antibodies to determine whether T cells are involved in mediating mIL-21-induced tumor regression. mIL-21-treated mice depleted of either $CD4^+$ or $CD8^+$ T cells still exhibited significant inhibition of MCA205 tumor growth, suggesting that T cells are not involved in IL-21 antitumor activity in this model (Fig. 5, *A*, *B*, and *D*). Tumor growth rate was noticeably higher in CD8-depleted mice compared with either CD4-depleted mice or control mice in the absence of mIL-21 (Fig. 5, *A*, *B*, and *D*), indicating that endogenous $CD8^+$ T cells may have some inhibitory effects on the baseline tumorogenicity of MCA205, a weakly immunogenic tumor line. Nevertheless, with the addition of mIL-21 plasmid, MCA205 tumor growth was significantly inhibited, suggesting that IL-21 could work mainly through a CD8-independent mechanism. Indeed, as shown in Fig. 5*C*, the antitumor activity of mIL-21 was completely abolished

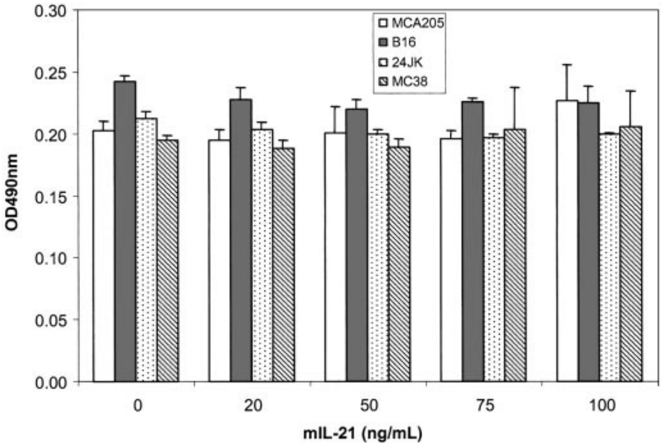

Fig. 4. mIL-21 does not inhibit tumor growth *in vitro*. The 1×10^5 tumor cells/well were plated onto 24-well plates, and various amounts of recombinant mIL-21 protein at the indicated concentrations were added to each well and incubated for 3 days. The culture medium from each well was then collected for a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium-based cell proliferation assay. Data represent one of three independent experiments with similar results. The *columns* are the mean values of triplicates in each condition with SDs.

Table 2 *Multiple cytokine secretion in serum from mice that received injections of mIL-21*

C57BL/6 mice received i.v. injections of either saline alone, 20 μg of pORF, or mIL-21 plasmid DNA, respectively, on day 0. Mouse serum was collected 16 h and 4 and 8 days after DNA administration and subjected to multiple cytokine immunoassays (expressed as pg/ml serum). Three mice were in each group at each point. Positive controls were from serum samples collected from mice that received injections of 1 μg of mIL-2, mIL-4, mIL-10, and mIL-12 plasmid DNA, respectively. Data represent one of two experiments with similar results.

Treatment	Day	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IFN-γ	TNFα
Saline	16 h	108 ± 24	258 ± 7	20 ± 4	68 ± 35	160 ± 29	34 ± 10	38 ± 6	33 ± 7	50 ± 21
pORF	16 h	137 ± 57	288 ± 62	24 ± 9	86 ± 45	308 ± 204	335 ± 469	48 ± 21	51 ± 24	75 ± 37
mIL-21	16 h	155 ± 60	297 ± 34	28 ± 6	111 ± 37	296 ± 54	248 ± 32	46 ± 9	60 ± 8	112 ± 51
None	16 h	89 ± 31	275 ± 11	18 ± 1	43 ± 19	206 ± 158	49 ± 40	43 ± 8	50 ± 36	307 ± 488
Saline	4	161 ± 83	393 ± 102	39 ± 9	151 ± 61	279 ± 149	85 ± 36	55 ± 20	70 ± 16	139 ± 44
pORF	4	161 ± 36	292 ± 73	25 ± 7	66 ± 17	124 ± 27	47 ± 18	53 ± 8	49 ± 8	78 ± 45
mIL-21	4	185 ± 138	306 ± 66	31 ± 11	82 ± 44	154 ± 76	276 ± 146	58 ± 32	50 ± 26	81 ± 31
Saline	8	232 ± 281	205 ± 170	22 ± 15	95 ± 47	146 ± 86	44 ± 24	36 ± 25	40 ± 30	83 ± 36
pORF	8	137 ± 20	276 ± 27	28 ± 4	90 ± 19	130 ± 15	50 ± 9	44 ± 24	48 ± 11	78 ± 22
mIL-21	8	154 ± 111	236 ± 177	24 ± 15	95 ± 15	834 ± 1269	413 ± 489	52 ± 37	253 ± 375	98 ± 30
mIL-2	16 h	216	36935	50.8	532.8	1965	116.7	25	101.2	75
mIL-4	16 h	69	61	1427	161	1820	550	23	71	142
mIL-10	16 h	49	176	40815	224	733.8	14935	27	270	681
mIL-12	16 h	56	30	8.8	822	351	84	3990	222.1	67

after *in vivo* depletion of NK cells. These experiments demonstrate that the inhibitory effect of mIL-21 on MCA205 tumor requires NK cells. This was further supported by a similar experiment using either CD4 or CD8 knockout mice that showed that mIL-21 could induce tumor regression in the absence of $CD4^+$ or $CD8^+$ T cells (data not shown). However, it is possible that $CD8^+$ T cells play a partial role in this antitumor effect because tumor growth rate in mIL-21-treated mice is greater in CD8-depleted mice compared with control mice (Fig. 5, *B* and *D*).

To determine whether splenic NK cells have specific cytolytic activity against tumor cells, we performed ^{51}Cr-release assay against B16 tumor targets, which express low levels of MHC class I molecules, using DX5-enriched splenic NK cells. As shown in Fig. 6*A*, the cytolytic activity of enriched NK cells from mIL-21-treated mice against B16 targets was significantly increased compared with NK cells from pORF-treated mice. The cytolytic activity of NK cells against YAC-1 targets was also significantly higher in mIL-21-treated mice than in pORF-treated mice (Fig. 6*B*). Because YAC-1 cells are more sensitive targets for NK cells, the extent of lysis against YAC-1 targets in both groups were relatively higher than that observed in B16 targets. To determine whether activated T cells and allospecific cytotoxic T cells attribute to the tumor killings, we also tested the cytolytic activity of splenic $CD8^+$ T cells against target cells. As shown in Fig. 6, *A* and *B*, $CD8^+$ T cells enriched from mice treated with either mIL-21 or control DNA showed no cytolytic activity against either B16 or YAC-1 targets. This result further confirmed that

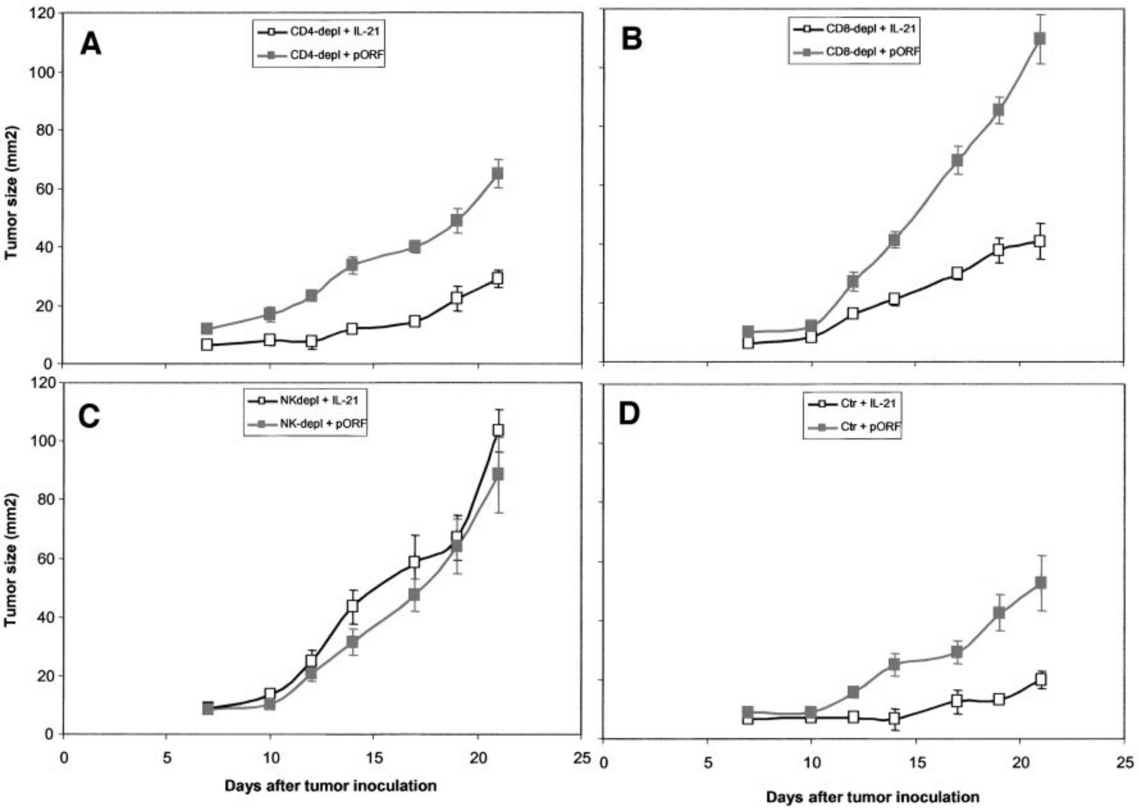

Fig. 5. Antitumor effect of mIL-21 in CD4-, CD8-, and NK-depleted mice. C57BL/6 mice received s.c. inoculations of 5 × 10^5 MCA205 tumor cells on day 0. Antibodies against either CD4, CD8, or NK cells were administered on days 2 and 4, respectively. The depletion was maintained by repeated injection of antibodies every 6–7 days thereafter throughout the entire experiment. Treatment of mIL-21 began on day 5 and was repeated once 1 week later. *A*, CD4-depleted mice. *B*, CD8-depleted mice. *C*, NK-depleted mice. *D*, control mice. Six mice were in each group. Data represent one of three similarly executed experiments with similar experiments. *Bars*, SE.

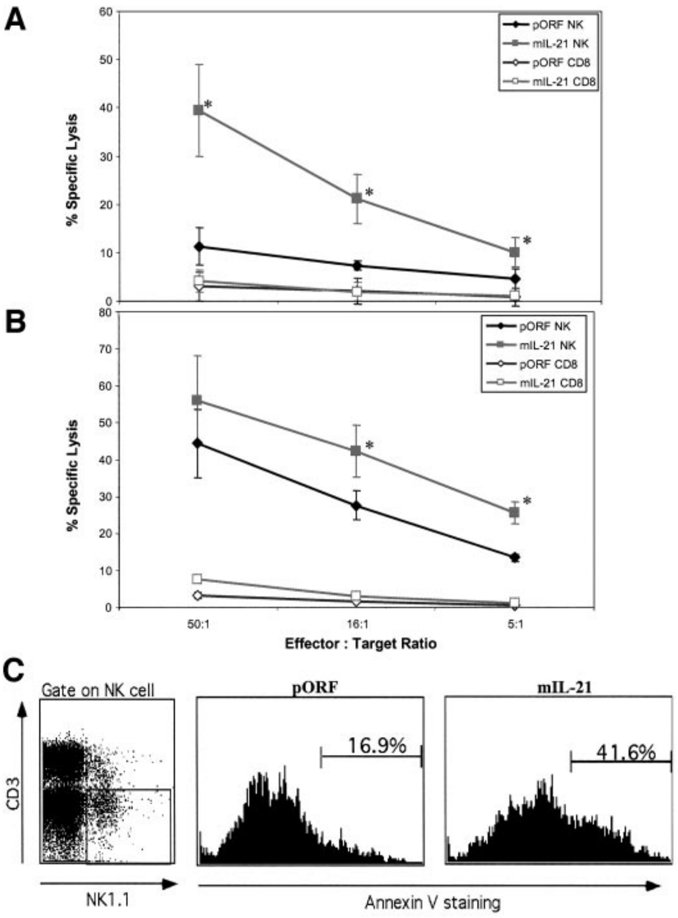

Fig. 6. IL-21 enhances NK cell apoptosis and cytolytic activity *in vivo*. *A*, enriched NK and CD8$^+$ T cells were incubated with ^{51}Cr-labeled B16 target cells to determine the cytolytic activity of NK cells. *B*, enriched NK and CD8$^+$ T cells were incubated with ^{51}Cr-labeled YAC-1 target cells to determine the cytolytic activity of NK cells. The results represent the mean of two independent experiments, each with five mice in each group. *C*, freshly isolated splenocytes from mice 4 days after either pORF or mIL-21 plasmid injection were stained with annexin V gated on NK1.1$^+$/CD3$^-$ NK cells to determine the apoptosis. Data represent one of five independent experiments with similar results. *, significant difference between groups ($P < 0.05$).

the *in vivo* antitumor activity in this animal tumor model was mainly mediated through NK cells rather than by T cells.

Interestingly, as shown in Table 1, the actual percentage and total number of NK1.1$^+$/CD3$^-$ or DX5$^+$/CD3$^-$ splenic NK cells decreased after mIL-21 injection compared with mice that received injections of the pORF control vector. To further investigate the mechanism of this decrease, we assessed NK cell apoptosis after *in vivo* mIL-21 plasmid injection. As shown in Fig. 6*C*, annexin V staining of NK1.1$^+$/CD3$^-$ splenic NK cells increased from 16.9 to 41.6% 4 days after mIL-21 plasmid injection, suggesting that mIL-21 had an apoptotic effect on NK cells *in vivo*. Taken together, these results demonstrate that IL-21 *in vivo* can both enhance NK cell activation and its lytic activity against tumor cells while inducing NK cell apoptosis, which helps to explain the observed NK-dependent antitumor activity in mIL-21-treated mice despite a reduction in the number of splenic NK cells.

DISCUSSION

Recent advances have highlighted the potential of immunotherapeutic approaches for cancer (14). Cytokine treatment remains a primary modality of tumor immunotherapy by activating immune cells *in vivo*. Systemic IL-2 administration can mediate long-term complete responses in some patients with widespread metastatic melanoma and renal cell cancer. However, the toxicity of IL-2 treatment has limited its clinical use. Therefore, we have sought to evaluate other potential cytokines to stimulate effector cells *in vivo*.

IL-21 has high sequence homology to IL-2 and IL-15, and its receptor binding protein, IL-21 receptor, is most like IL-2 receptor β (5, 7). Moreover, like IL-2 and IL-15, the receptor also shares the common cytokine receptor γ-chain γc (15, 16). IL-21 plays an important role in regulating T-cell, B-cell, and NK cell functions (5, 7, 8), and IL-4 and IL-21 together critically regulate immunoglobulin production (17). In combination with IL-2 and Flt3L, IL-21 has been shown to enhance the proliferation and differentiation of NK cells from human CD34$^+$ bone marrow progenitors and augments the effector lytic function of NK cells against K562 target cells (5). Kasaian *et al.* (8) subsequently reported that IL-21 has an inhibitory effect on the IL-15-mediated expansion of resting mouse NK cells and that IL-21 enhanced cytotoxic activity in NK cells, previously activated by polyriboinosinic polyribocytidylic acid *in vivo* or IL-15 *in vitro*, but does not induce activation of resting NK cells (8). IL-21 has also been shown to induce the apoptosis of resting and activated primary murine B cells (18). Our studies have demonstrated that, in mice receiving mIL-21 plasmid DNA, IL-21 also promoted apoptosis in NK cells while dramatically inducing enhanced cytolytic activity of NK cells against target cells such as B16 and YAC-1 (Fig. 6).

In this study, we have used a hydrodynamics-based gene delivery technique to generate sustained production of large amounts of circulating IL-21 protein *in vivo* to treat established s.c. tumors. Although this method may not be practical in the clinic, it allows us to effectively and efficiently study the *in vivo* biological effects of cytokines in small animals without producing large amounts of recombinant protein that is often a laborious, time-consuming, and expensive procedure limiting research. We have demonstrated that administration of mIL-21 plasmid DNA could inhibit tumor growth *in vivo* and this antitumor activity was unaffected by depletion of CD4$^+$ T cells and, at most, only partially affected by depletion of CD8$^+$ T cells. However, depletion of NK cells completely eliminated antitumor activity, indicating that they are required for the antitumor effect of IL-21. It is conceivable that the observed *in vivo* antitumor activity of IL-21 is because of enhanced cytolytic activity of NK cells after IL-21 injection (Fig. 6), although the percentage and total number of NK cells in the spleen and peripheral blood were decreased (Table 1), possibly because of the enhanced NK cell apoptosis after IL-21 that we observed. Given the fact that the percentage and the absolute number of NK cells in IL-21-treated mice were decreased, the actual cytolytic activity of NK cells on a single-cell basis might be even higher. Indeed, the ^{51}Cr-release assay using enriched NK cells has confirmed that NK cells from IL-21-treated mice had a much stronger lytic activity against B16 tumor targets. Enriched CD8$^+$ T cells from IL-21-treated mice did not exhibit any cytolytic activity against target cells in the lysis assay, further supporting our conclusion that it is NK cells that may serve an important effector in the suppression of tumor growth, and that T cells may not play a major role in this model. Using genetically engineered B16F1 tumor cells expressing IL-21 and NK-depleted mice, Ma *et al.* (19) recently demonstrated that NK cells are required for the rejection of B16F1-IL-21 tumors, although they did not address the effect of systemic IL-21 on untransduced, wild-type tumors. Whereas the use of cytokine-transduced tumors may provide insight into the effects of the cytokine on the immunogenicity of the transduced cells (20, 21), this approach does not provide information regarding the effectiveness of systemic cytokine levels on wild-type tumors, which is essential for clinical application. It remains unclear at this point whether increases in cells of the myelomonocytic lineage after IL-21 injection, as evidenced by increases in CD11b$^+$, CD11c$^+$, and Gr-1$^+$ cell subpopulations, contribute to IL-21-induced antitumor activity.

Rapid injection of plasmid DNA has been shown in some studies to induce expression of IL-12 and TNF-α (13), which could indirectly contribute to tumor regression. In this study, however, we saw no consistent increases in serum levels of 10 cytokines, including IL-12 and TNF-α, at the time points of 16 h and 4 and 8 days after injection of either control or mIL-21 plasmid (Table 2). Therefore, it is unlikely that the mIL-21 plasmid induced cytokines that mediated tumor regression. Rather, the effect is more likely attributable to direct activation of NK cells.

Although IL-2 has potent effects on NK cells (22–24), we found that it did not have any antitumor activity in this model. This is possibly explained by the fact that we could only administer limited amounts of IL-2 plasmid by this method because of the high level of toxicity. Increasing the IL-2 dose caused a significant number of mice to die from the cytokine, perhaps because of the release of secondary cytokines, as seen in Table 2. However, IL-21 had little toxic effects on mice, did not induce other cytokines, and was well tolerated at doses as high as 20 μg of plasmid. We hypothesize that this difference in tolerated dose may explain the NK-mediated antitumor effects seen with IL-21 but not IL-2, despite the fact that the latter has well described effects on NK cells.

This study presents the first evidence that systemic administration of IL-21 can activate immune cells to mediate tumor regression *in vivo*. Importantly, we saw no toxicity in mice expressing high levels of circulating IL-21, which is often a crucial limiting factor in consideration of the clinical use of cytokines. However, it is not clear whether there would be any toxicity associated with even higher levels of IL-21 or with long-term administration, both of which can be tested using recombinant IL-21 protein. In addition, because cytokines such as IL-2, IL-12, IL-15, and IFN-γ have been shown to enhance the cytolytic activities and antitumor functions of NK cells (25–28), it will be of interest to determine whether the effectiveness of IL-21 in suppressing tumor growth *in vivo* can be further enhanced in combination with these cytokines or whether IL-21 can be used to enhance the antitumor activity of adoptively transferred T lymphocytes. These experiments are currently under investigation.

In summary, our study demonstrates that IL-21 can inhibit the growth of solid tumors in animal models through a NK cell-dependent mechanism and may be an important cytokine to test for therapeutic efficacy in cancer patients.

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In Vivo Antitumor Activity of Interleukin 21 Mediated by Natural Killer Cells

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