Adenovirus-Interleukin-12-mediated Tumor Regression in a Murine Hepatocellular Carcinoma Model Is Not Dependent on CD1-restricted Natural Killer T Cells


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ABSTRACT

The cytokine interleukin-12 (IL-12) has shown potent antitumor activity in several tumor models. Recently, natural killer (NK) T cells have been proposed as mediator of the antitumor effects of IL-12. In this study, the antitumor response of IL-12 was investigated in a gene therapeutic model against s.c. growing mouse hepatocellular carcinomas using an adenoviral vector expressing murine IL-12 (AdVmIL-12). An adenoviral-based system was chosen because of the ability of adenoviruses to transduce dividing and nondividing cells and because of their high transduction efficiencies. Our goals were to examine the efficacy of AdVmIL-12 in a hepatocarcinoma mouse tumor model and to investigate the mechanism of the AdVmIL-12-mediated antitumor response with specific interest in the role of NK T cells. Our studies demonstrate that intratumoral AdVmIL-12-mediated regression of s.c. hepatocellular tumors is associated with rapid antitumor responses. AdVmIL-12 treatment was associated with an immune cellular infiltrate consisting of CD4 and CD8 T lymphocytes, macrophages, NK cells, and NK T cells. Antibody ablation of CD4 and CD8 T cells and use of NK cell-defective beige mice failed to abrogate the response to AdVmIL-12. Studies in T-cell- and B-cell-deficient severe combined immunodeficient and recombinase activating gene-2-deficient mice and T-cell-, B-cell-, and NK cell-defective severe combined immunodeficient/beige mice also failed to abrogate this response. AdVmIL-12 retained potent antitumor activity in mice with specific genetic defects in immune cellular cytotoxicity (perforin knockout mice) and costimulation (CD28 knockout mice). Use of mice with specific NK T cell deficiencies, Vα14 T-cell receptor and CD1 knockout mice, also failed to abrogate the response to AdVmIL-12. Histological and immunohistochemical studies of AdVmIL-12-treated tumors showed extensive inhibition of neovascularization and a marked decrease in factor VIII-stained endothelial cells. Our studies indicate that the antitumor response of AdVmIL-12 is independent of direct cytotoxic cellular immunity (specifically, the function of NK T cells) and suggest that the initial mechanisms of AdVmIL-12-mediated tumor regression involve inhibition of angiogenesis.

INTRODUCTION

The heterodimeric cytokine IL-12 has been shown to generate powerful antitumor responses in many tumor models. IL-12 is the key cytokine mediating the generation of a Th1-type cytokine expression pattern in both T lymphocytes and NK cells, resulting in cytotoxic cellular immune responses (1–5). IL-12-activated cells preferentially secrete IFN-γ, which has been identified as an important downstream mediator of the IL-12 antitumor response (5–13). Although the immune mechanisms stimulated by IL-12 have been studied extensively, there is considerable controversy regarding the specific mechanisms by which IL-12 inhibits tumor growth. Investigations into the precise molecular and cellular events that mediate tumor regression after IL-12 treatment have been fueled by the demonstration of higher in vivo efficacy compared with other antitumor cytokines (13–15).

Studies examining the antitumor mechanisms of IL-12 have implicated various cellular mediators including CD8 T cells, NK cells, and NK T cells (5, 13, 16, 17). NK T cells are a recently described T-cell subset that recognizes a limited array of peptide and nonpeptide antigens presented by the nonpolymorphic MHC-like molecule CD1 (18, 19). NK T cells express a limited repertoire of TCR genes [mostly Vα14 in mice and Vα24 in humans (19, 20)]. NK T cells readily respond to cytokine stimulation (IL-12) and to ligand-mediated activation [α-galactosylceramide (21, 22), α-Galactosylceramide, a glycolipid with the unique ability to specifically activate Vα14 NK T cells, has been shown to stimulate NK T cell-mediated antitumor responses that are dependent on CD40-CD40 ligand and B7-1/CD28 interactions and on perforin-mediated cytotoxicity (22–24)]. In one tumor model, there was an absolute requirement of NK T cells for IL-12-mediated antitumor responses, and perforin-dependent cytotoxicity was suggested to mediate the response (25). In another study, NK T cells were shown to be required for both IL-12-mediated IFN-γ production and tumor-associated cytotoxicity (26).

Cytokine gene therapy is becoming a promising weapon in the armamentarium against cancer (27). Adenoviral-based cytokine gene therapy has many advantages over other forms of cytokine delivery (28). Adenoviral vectors allow local, high-efficiency, but transient transgene expression, generating high-level but self-limited cytokine responses in treated tumors. Adenoviral vectors are capable of transducing nondividing cells, increasing the number of transduction targets in a heterogeneous growing population of tumor cells.

We tested AdVmIL-12 in a murine HCC model. Our goals were to test the efficacy of AdVmIL-12 against hepatocellular tumors and to elucidate the mechanisms mediating this response. We were specifically interested in determining the requirement for NK T cells and in identifying possible non-immune mechanisms that contribute to the response. Our studies demonstrate that cytotoxic immune effector mechanisms are not required for AdVmIL-12-mediated antitumor responses in murine HCC models and suggest that AdVmIL-12 mediates tumor regression by inhibition of angiogenesis.

MATERIALS AND METHODS

Viruses. Construction of the adenovirus murine-IL-12.1 vector (AdVmIL-12), a generous gift from Dr. Frank Graham (McMaster University, Ontario, Canada), has been described previously (29). This vector contains the p35 and...
p40 subunit cDNAs of murine IL-12 in the early regions 1 (E1) and 3 (E3), respectively of adenovirus type 5. The genes are driven by the human cyto-
megalovirus immediate early gene promoter/enhancer. Adenovirus-luciferase (AdVLuc), an E1-deleted and replication-deficient adenovirus type 5 vector, was generously provided by Dr. Michael Barry (University of Texas-South-
western, Dallas, TX). AdVLuc contains, in the former E1 site, the firefly Photinus pyralis luciferase reporter gene driven by the cymomegalovirus pro-
moter/enhancer. CsCl gradient-purified vector was titrated on 293 human embryonal kidney cells (30). Working stocks of the virus were propagated on 293 cells.

Mice and Cell Lines. The 6–9-week-old C57BL/6, C171 SCID, C171 SCID/beige, and C129/RAG-2-immune-deficient mice were bred and main-
tained in a specific pathogen-free colony at the Experimental Radiation On-
cology Facility at UCLA. NK cell-defective C57BL/6/beige, C57BL/6/perforin-knockout, and C57BL/6/CD28-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6/V,14 NK T cell-defic-
tent mice were the generous gift of Dr. Masaru Taniguchi (Chiba University, Chiba, Japan). C57BL/6/CD1-deficient mice were the generous gift of Dr. Luc Van Kaer (Vanderbilt University, Nashville, TN). All mice obtained from
outside sources were bred and housed in a quarantine area in the specific
pathogen-free colony at UCLA. All animal studies were conducted in accord-
ance with the UCLA Animal Care Policy as prescribed by the Chancellor’s Animal Research Committee. BWIC3 and its derivative cell line Hepa 1-6 are well-characterized murine HCC lines, and B16 is a well-characterized murine
melanoma cell line (31, 32). All cell lines were obtained from the American
Type Culture Collection (Manassas, VA). Hepa 1-6 was maintained in vitro in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with 10% FCS (Gemini Products, Calabasas, CA) and 1% (v/v) penicillin, streptomycin,
and fungizone (Gemini Products; complete media). BWIC3 and B16 were main-
tained in vitro in DMEM (Life Technologies, Inc.) complete media. Human
embryonal kidney 293 cells provided by Dr. Kohnosuke Miti (UCLA, Los
Angeles, CA) were passaged similarly in DMEM media. Tumors for in vitro
studies were maintained through serial passage of single cell suspensions in
the subcutis of mice as described previously (33). Tumors used to generate single
cell suspensions were resected from the s.c. position in euthanized mice,
mechanically dispersed, and enzymatically digested for 2 h with DmNase I (0.1
mg/ml; Sigma) and collagenase D (1 mg/ml; Boehringer Mannheim, Indian-
apolis, IN) in 40 ml of AIM-V media (Life Technologies, Inc.). The single cell
susensions were then washed three times with PBS and either injected into
mice to maintain in vivo tumor or used for in vitro studies.

Treatment and Monitoring of Established Tumors. All tumors were
deciphered in the dorsal flanks of mice by s.c. injection of serially passaged
cells (4–5 × 10^6 cells/injection) suspended in PBS. After 1–2 weeks of in vivo
growth, tumor-bearing mice were randomized into groups with size-matched
tumors (average mean tumor volume, 40–200 mm^3) and treated intratumorally with either AdVmIL-12 or PBS as a control. As a positive control, tumor-
bearing wild-type mice were treated in parallel with either AdVmIL-12 or PBS.
Tumor growth was assessed by calipers, taking the mean of two perpen-
dicular diameters at the time of intratumoral therapy and every 3–4 days
thereafter. Tumor volume was estimated by the formula 4/3 π r^3.

In Vivo Depletion of CD4 and CD8 T-Cell Subsets. In vivo monoclonal
antibody ablation of CD8 (clone 2.43; ATCC TIB 210) or CD4 (clone GK1.5;
ATCC TIB 207) T-cell subsets was performed by i.p. injection on days 5, 3,
and 1 before tumor inoculation and every 7 days thereafter (0.5 mg antibody/
mouse/injection). Antibody suspensions were prepared from hybridoma super-
nants by passage through protein G columns according to the manufacturer’s
instructions (Pierce, Rockford, IL). Eluted immunoglobulins were dialyzed
against PBS and stored at 4°C in 1 mg/ml suspensions. CD4 and CD8 T-cell
depletion was confirmed by flow cytometric analysis of splenocytes from
depleted mice on the day of tumor challenge.

Splenocyte Harvest. Spleens were harvested from euthanized mice, me-
chanically dispersed, and treated with ACK buffer (0.15 M NH_4Cl, 10 mM
KHCO_3, and 0.05 mM NaEDTA) to deplete RBCs. The RBC-depleted spleno-
cyte populations were then resuspended and washed three times in PBS.
Washed cells were then used for additional studies.

Flow Cytometric Analysis. Splenocyte populations and tumor single cell
suspensions were obtained as described above. The following preconjugated monoclonal antibody antibodies were used: (a) anti-CD4-FITC (Caltag, Bur-
ingame, CA); (b) anti-CD8-PE (PharMingen, San Diego, CA); (c) anti-
NK1.1-PE (PharMingen); and (d) anti-CD3-FITC (PharMingen). For NK T cell
identification, cells were double stained with anti-NK1.1 and anti-CD3.

After harvest, splenocytes and tumor samples were resuspended and washed
twice in cold PBS with 2% FCS. Antibody staining of splenocytes and tumor samples (5 × 10^6 cells/sample) was performed in 5–10 μl of antibody solution
for 1 h. At the end of the incubation period, the stained populations were
washed three times in cold PBS with 2% FCS and then resuspended in 1%
parafomaldehyde fixative. Flow cytometric analysis was performed on a
FACScan machine (Becton Dickson, San Jose, CA).

Histological Analysis. For histological studies, AdVmIL-12-treated and
PBS-treated tumors were harvested, fixed in formalin, embedded in paraffin,
sectioned, stained with H&E, and examined under a light microscope. For
immunohistochemical staining, formalin-fixed paraffin-embedded blocks were
sectioned at 2μm and placed on positively charged slides. Slides were baked
at 60°C for 30 min, deparaffinized in xylenes, and rehydrated to tap water
through graded ethanol. Slides were then treated with 3% hydrogen peroxide
in methanol for 10 min to quench endogenous peroxidase activity. The sections
were then subjected to heat-induced antigen retrieval in 0.01 M citrate buffer
(pH 6.0) for 25 min in a steamer (Black and Decker) and allowed to cool for
15 min before transferring to 0.01 M PBS. Immunostaining was performed on
da DAKO Autostainer (DAKO Corp., Carpinteria, CA) using the Envision+
Rabbit Peroxidase Detection System (DAKO Corp.). The heat-treated slides
were incubated with anti-factor VIII (rabbit anti-von Willebrand factor; DAKO
Corp.) primary antibody (diluted 1:200) for 45 min. After primary antibody
treatment, the slides were rinsed in PBS and incubated with Envision+
anti-rabbit peroxidase for 30 min. The antibody was then visualized with
diaminobenzidine as the chromogen and counterstained with Harris hemato-
xylin.

Quantification of Tumor Vascularity. Eleven days after treatment, Ad-
VmIL-12-treated and PBS-treated tumors were harvested, fixed in formalin,
and stained with H&E. Slides were then examined under a light microscope,
and the number of blood vessels per ×200 field was counted. Blood vessel
counts/field were then averaged.

Statistical Analysis. Student’s t test was performed to interpret the signif-
cance between the final tumor volumes of animals treated with AdVmIL-12
and those treated with PBS. Two-sided P values respect individual comparisons.

Tumor growth and vascularity data represent the mean ± SE.

RESULTS

In Vivo Efficacy of AdVmIL-12. The antitumor effect of Ad-
VmIL-12 was tested in the closely related BWIC3 and Hepa 1-6 cell
lines. AdVmIL-12 was shown to have potent antitumor effects when used
to treat established hepatocellular tumors. A representative study is shown in Fig. 1 in which a single intratumoral injection of 1 × 10^9
pfu of AdVmIL-12 into BWIC3 tumors (average tumor volume, 200
mm^3) induced a rapid antitumor response. Increasing the viral dose resulted in more profound antitumor responses, but doses above 6 × 10^9
pfu (as a single injection) were uniformly associated with systemic toxicity and death. These general observations were drawn
from a total of 24 studies using 178 mice. Intratumoral AdVmIL-12
mediated partial regression in 90% of mice and complete regressions
in 10% of mice. Antitumor responses to intratumoral AdVmIL-12
treatment were similar in BWIC3 and Hepa 1-6 tumors. All mice that
achieved complete tumor regressions were protected from a rechal-
lenge with the same tumor at 8 weeks, suggesting the generation of
tumor-specific immunity.

Cellular Infiltrates in AdVmIL-12-treated Tumors. We next examined the tumor infiltrate to gain insight into the cellular require-
ments of the AdVmIL-12-mediated antitumor response. Flow cyto-
metric analysis of single cell suspensions of AdVmIL-12-treated and
PBS-treated tumors demonstrated a large host cell infiltrate associated
with AdVmIL-12 treatment (Fig. 2). Analysis of the total number of
cells in the tumor sample revealed a dramatic increase in the number of
CD4, CD8, NK, and NK T cells and a decrease in the number of
Similar results were seen in triplicate experiments. Tumors compared with both PBS-treated and AdVLuc-treated tumors (p = 0.009). Similar results were seen in triplicate experiments.

tumor cells in the AdVmIL-12-treated tumors as compared with the control.

**AdVmIL-12 Effectively Mediates Tumor Regressions in Mice with Specific Immunological Deficiencies.** Antibody ablation and genetically immunodeficient mice were used to determine the contribution of particular immune cell subtypes to the AdVmIL-12-mediated antitumor response. *In vivo* monoclonal antibody depletions of CD4 and CD8 T-lymphocyte populations failed to abrogate the response to intratumoral AdVmIL-12 treatment of established BWIC3 tumors (Fig. 3, A and B). AdVmIL-12 also retained potent antitumor activity in NK cell-defective beige mice (Fig. 3C), in T-cell- and B-cell-deficient SCID (Fig. 3D) and RAG-2-deficient (Fig. 3E) mice, and in SCID/beige (Fig. 3F) mice deficient in functional T, B, and NK cells. Thus, T, B, or NK cells are not required for AdVmIL-12-mediated antitumor activity.

**AdVmIL-12-mediated Antitumor Activity in Mice Devoid of NK T Cells.** The antitumor effects of IL-12 have been reported to be dependent on the presence of functional NK T cells (25, 26). This hypothesis was tested in two different mouse strains harboring NK T-cell-deficient phenotypes: (a) CD1 knockout mice; and (b) Vα14 NK T-cell knockout mice. The lack of Vα14 or CD1-dependent (Fig. 4B) NK T cells failed to abrogate the response to intratumoral AdVmIL-12 treatment of Hepa 1-6 tumors. Therefore, AdVmIL-12-mediated antitumor responses appear to be independent of the effector functions of NK T cells.

**Effect of AdVmIL-12 in Mice with Defects in Immune Cell Cytostimulation and Cytotoxicity.** Specific deficiencies in cellular immune compartments may still fail to determine the AdVmIL-12 antitumor mechanism if several cell types can mediate the response. Therefore, we used mice with deficiencies in common immune mechanisms required for the generation of immune responses and effector mechanisms, namely, CD28 costimulation and perforin-mediated cytotoxicity, respectively. The perforin pathway is a final common mediator of NK, NK T, and CD8 T-lymphocyte cytotoxic effects, and T-cell-associated CD28 interactions with antigen-presenting cell-associated B7-1 are a crucial costimulatory pathway in the generation of cell-mediated responses (25, 34, 35). AdVmIL-12 effectively generated regression of hepatocellular tumors in CD28 and perforin knock-out mice (Fig. 5, A and B). Thus, common immune cell costimulatory and cytotoxic effector mechanisms are not required for the generation of AdVmIL-12-mediated antitumor responses.

**AdVmIL-12-mediated Antiangiogenic Effects.** We finally explored the possibility that AdVmIL-12 initiates antitumor responses by a mechanism independent of direct cytotoxic cellular immunity. AdVmIL-12-treated tumors stained with H&E clearly demonstrate a decrease in tumor vascularity evidenced by a statistically significant decrease in the number of intratumoral blood vessels 11 days after treatment compared with PBS-treated controls (Fig. 6C). H&E-stained AdVmIL-12-treated tumors show decreased blood vessel formation and extensive cellular necrosis compared with an abundance of blood vessel infiltration and rich tumor growth in the PBS-treated tumors (Fig. 6B). This is in marked contrast with staining of PBS-treated tumors (Fig. 6B), which clearly demonstrates neovascular endothelial cell staining with anti-factor VIII antibody. The marked decrease in neovascularization of AdVmIL-12-treated tumors suggests that antiangiogenic mechanisms initiate the antitumor responses produced by intratumoral AdVmIL-12.

**DISCUSSION.** Our goals in this study were to determine the efficacy of adenoviral-based IL-12 gene therapy in murine HCC models and to elucidate the mechanism of the antitumor response with particular interest in the role of NK T cells. We demonstrate that intratumoral AdVmIL-12 administration is effective in producing antitumor responses in small established hepatocellular tumors. All animals had statistically significant tumor regression, with 10% of animals showing complete tumor eradication. The therapeutic index was relatively narrow, with higher and more effective viral doses causing systemic toxicity. Because IL-12 plays a profound role in the regulation of cellular immunity, it was interesting that this arm of the immune system did not appear to mediate the initial phase of tumor regression. AdVmIL-12 was equally effective in settings in which such effector cells were either ablated or genetically deficient. CD4, CD8, and NK cells were not required for the antitumor response of intratumoral AdVmIL-12. Although NK T cells are a specific target of IL-12, they do not appear to mediate the antitumor effects of AdVmIL-12. AdVmIL-12 was also effective in mice with a targeted gene mutation in perforin, a common mediator of NK, NK T-cell, and T-cell cytotoxicity (25, 34). The preservation of AdVmIL-12 efficacy in mice with a targeted gene mutation in CD28, an important costimulatory molecule, strongly supports the immune effector cell independence of this antitumor response (35). Thus, it appears that intratumoral AdVmIL-12 treat-
ment of murine hepatocellular tumors is associated with rapid and potent antitumor responses that are independent of NK T cells and cell-mediated immunity.

Various investigators have explored the antitumor effect of direct intratumoral AdVmIL-12 treatment of established tumors. Transgenic mice developing spontaneous breast tumors given a single intratumoral injection of AdVmIL-12 showed tumor regressions in 75% of treated mice (36). Notably, IFN-γ was produced in significant quantities within IL-12-treated tumors. Complete responders rejected a subsequent rechallenge, suggesting the development of immunological memory. Although it was not directly explored in the current studies, the mechanism of this immunological memory may be cross-presentation of tumor-derived antigenic epitopes released by dying tumor cells, leading to a delayed immunological response. In another study, intratumoral AdVmIL-12 was shown to significantly increase the survival of mice bearing intrahepatic MCA-26 tumors in a metastatic colon cancer model (37). A single intratumoral injection of AdVmIL-12 was able to generate tumor regressions in a dose-dependent manner in mice bearing murine bladder tumors, with mice receiving $1 \times 10^9$ pfu of virus consistently achieving complete growth inhibition.

**Fig. 3.** Effect of AdVmIL-12 in mice with specific immune cell subtype defects. A and B, effect of AdVmIL-12 in mice depleted of CD4 and CD8 T lymphocytes. C57BL/6 mice were treated i.p. with monoclonal antibody to CD4 or CD8 on days 5, 3, and 1 before tumor inoculation (BWIC3; $5 \times 10^6$ cells) and every 7 days thereafter ($n = 4$ mice/group). On day 7 after tumor inoculation (average tumor volume, 35–55 mm$^3$), mice were treated intratumorally (arrow) with $2 \times 10^9$ pfu of AdVmIL-12 or with an equivalent volume of PBS. Significant growth inhibition was seen in mice depleted of CD4 (A) and CD8 (B) T cells and in naive mice treated with AdVmIL-12 compared with PBS-treated controls ($*, P = 0.01; **, P = 0.005; +, P = 0.008$). Similar results were seen in duplicate studies. C–F, effect of AdVmIL-12 in genetically immunodeficient mice. Immunodeficient mice (as identified in the figure) were inoculated in the right flank with $5 \times 10^6$ BWIC3 tumor cells on day 0 ($n = 4$ mice/group). On days 5, 8, 10, and 11, respectively, beige (C), SCID (D), RAG-2-deficient (E), and SCID/beige (F) mice were treated intratumorally (arrow) with either $2 \times 10^9$ pfu of AdVmIL-12 or an equivalent volume of PBS. Significant tumor regressions were seen in all immunodeficient mouse backgrounds after AdVmIL-12 treatment compared with PBS-treated controls ($*, P = 0.0002; **, P = 0.001; +, P = 0.0002; +++, P = 0.01$). Similar results were seen in duplicate studies. Although not shown in the graphs, wild-type tumor-bearing mice were also treated with either AdVmIL-12 or PBS, with results similar to those shown in Figs. 1, 4, and 5.
Successively passaged Hepa 1-6 tumor cells (5 × 10⁵) were inoculated with 5 × 10⁴ Hepa 1-6 tumor cells on day 0 (n = 5 mice/group). On days 5 and 9, respectively, Vα₁₄ NK T-cell knockout (A) and CD1 knockout (B) mice were treated intratumorally (arrow) with 2 × 10⁶ pfu of AdVmIL-12 or an equivalent volume of PBS. AdVmIL-12 and PBS-treated wild-type mice, treated identically as described for knockout mice, were followed concurrently in each study for comparison. Significant tumor regressions were seen in wild-type mice and both NK T-cell knockout backgrounds after AdVmIL-12 treatment compared with PBS-treated controls (*, P = 0.01; **, P = 0.0001; †, P = 0.0003; ‡, P = 0.0001). These results were consistent in duplicate studies.

Responses (16). Complete responders rejected a subsequent rechallenge. These antitumor responses were shown to be systemic by experiments in which contralateral un.injected tumors regressed after intratumoral AdVmIL-12 treatment of similar tumors growing in the opposite flank. Intratumoral AdVmIL-12 was also shown to generate tumor regressions in a s.c. colon cancer (CT-26) model with 76% of treated mice achieving a complete response, and complete responders also rejected a subsequent rechallenge (38). Nasu et al. (39) examined the effect of intratumoral AdVmIL-12 on the growth of orthotopically placed prostate tumors and showed significant tumor regressions, decreased numbers of pre-established pulmonary metastases, and prolonged survival in AdVmIL-12-treated mice.

NK T cells have been proposed as a requisite cell type in IL-12-mediated tumor regression. Kawamura et al. (26) showed that IL-12-stimulated liver and spleen mononuclear populations from mice with a targeted gene mutation in CD1 were less cytotoxic against YAC-1 and P815 tumor cell lines than against wild-type mononuclear cells after i.p. injection of recombinant IL-12. CD1 is a MHC-like molecule required for positive selection of CD1-dependent NK T cells during immune cell ontogeny (19). This group also showed that serum IFN-γ levels were lower in CD1-deficient mice than in wild-type mice after i.p. IL-12. These data are contrary to our data that show retention of AdVmIL-12 antitumor activity in the same CD1-deficient mice. Cui et al. (25) and Taniguchi et al. (40) examined the effect of recombinant IL-12 in mice with a targeted mutation in the Vα₁₄ TCR gene that preferentially lack Vα₁₄ NK T cells, the predominant NK T-cell subtype. Their studies showed that the antitumor effect of i.p. recombinant murine IL-12 against B16 was abrogated in Vα₁₄ NK T-cell-deficient mice and was restored in RAG mice genetically engineered to preferentially develop Vα₁₄ NK T cells but lack T, B, and NK cells (40). The authors suggested that there is an absolute requirement for NK T cells to mediate the antitumor effects of IL-12 because IL-12 efficacy was abrogated in Vα₁₄ NK T-cell-deficient mice and completely restored in mice harboring Vα₁₄ NK T cells as their only lymphoid cell type. This in vivo data are contrary to our data, although we used the same Vα₁₄ NK T-cell-deficient mice used in their study. In vitro studies showed that concanamycin A, a known inhibitor of perforin-mediated killing, could inhibit Vα₁₄ NK T-cell cytotoxicity (41). To test the hypothesis that Vα₁₄ NK T cells require perforin to mediate cytotoxic antitumor responses to IL-12, we used perforin...
MECHANISM OF AdVmIL-12-MEDIATED TUMOR REGRESSION

AdVmIL-12-treated

PBS-treated

Fig. 6. Vascularity of AdVmIL-12 treated and PBS-treated hepatocellular tumors. Eleven days after intratumoral treatment with either AdVmIL-12 or PBS, tumors were harvested from their s.c. position and fixed in formalin. After 24 h, the formalin-fixed tumors were embedded in paraffin, sectioned, and placed on positively charged slides. A, H&E-stained sections (>200) from AdVmIL-12-treated and PBS-treated tumors demonstrating marked blood vessel infiltration (arrow) in PBS-treated tumors and profoundly diminished neovascularization and distinct necrosis (hatched arrow) in AdVmIL-12-treated tumors. B, immunohistochemical analysis (>200) of AdVmIL-12-treated and PBS-treated tumors demonstrating factor VIII-stained neovascularization in PBS-treated tumors (arrow) with a marked decrease in factor VIII staining in AdVmIL-12-treated sections. C, blood vessel counts in H&E-stained sections from AdVmIL-12-treated and PBS-treated tumors. There is a statistically significant decrease in the number of blood vessels per high-power field (>200) in AdVmIL-12-treated tumors compared with PBS-treated tumors 11 days after treatment (*, P = 0.001).

knockout mice and were consistently able to generate potent antitumor responses to intratumoral AdVmIL-12. The discrepancy between these two reports and our data may be explained by a difference in study design and IL-12 delivery. Their studies, which examined the effect of recombinant IL-12 delivered systemically, differed dramatically from our studies using AdVmIL-12 delivered intratumorally with respect to the amount and duration of IL-12 presence in the treated tumors. The high-level of IL-12 produced in tumors treated intratumorally with AdVmIL-12 may directly initiate antiangiogenic mechanisms, obviating the need for specific cellular subsets to generate tumor regressions. The absolute requirement of NK T cells for IL-12 efficacy is not supported by our data.

NK T cells have the unique ability to respond to nonpeptide antigens, namely glycolipids, through their invariant TCR (22, 42). The glycolipid, \( \alpha \)-galactosylceramide, when presented in the context of the MHC-like molecule CD1, has been shown to specifically activate \( V_{a14} \) NK T cells (23, 43). This \( \alpha \)-galactosylceramide-mediated activation was shown to be dependent on \( B7-1/CD28 \) interactions (23). To test the possibility that \( V_{a14} \) NK T cells are stimulated in vivo by IL-12 and generate antitumor responses through glycolipid/CD1-dependent mechanisms, we used mice with a targeted gene mutation in CD28. In our studies, AdVmIL-12 effectively mediated antitumor responses in CD28 knockout mice, further supporting the NK T cell independence of the antitumor effects of IL-12.

Antiangiogenesis is an antitumor mechanism that nonspecifically impairs tumor growth by limiting access of vital nutrients and metabolites to rapidly growing tumors, leading to cellular apoptosis and tumor regression (44). Inhibition of angiogenesis is suggested in established tumors by the identification of poor tumor neovascularization on histological examination and by demonstrating an objective decrease in endothelial cell infiltration into tumors using immunohistochemistry and specific endothelial cell markers (45–48). Our studies show that treatment of established hepatomas with AdVmIL-12 results in marked inhibition of neovascularization with a distinct decrease in the number of blood vessels and factor VIII-staining endothelial cells in treated tumors.

The antiangiogenic effects of IL-12 have been well documented. One of the earliest reports of the antiangiogenic effects of IL-12 was by Voest et al. (49), who showed that recombinant murine IL-12 significantly inhibited corneal neovascularization in C57BL/6, SCID, and beige mice. IFN-\( \gamma \) antibody treatment abolished this effect, underscoring the importance of IFN-\( \gamma \) in IL-12-dependent antiangiogenesis. Notably, as in our studies, IL-12 was effective in SCID and beige mice, which argues for a similar antiangiogenic mechanism in our model. Since then, many reports have more clearly defined the mechanism by which IL-12 inhibits angiogenesis. Sgadari et al. (50) and Angiolillo et al. (51) showed that IL-12 induces the expression of IP-10 from mouse splenocytes, a known inhibitor of angiogenesis. In this study anti-IFN-\( \gamma \) antibody completely abolished the antiangiogenic effect of IL-12, whereas antibody to IP-10 resulted in significant but partial inhibition of IL-12-induced antiangiogenesis. These data suggest that IFN-\( \gamma \) is a requisite mediator of IL-12-mediated antiangiogenic effects, with IP-10 being an important downstream regulator of IFN-\( \gamma \)-dependent antiangiogenesis. In another study, Dias et al. (8) demonstrated, in a murine breast cancer model, that IL-12 downregulates vascular endothelial growth factor levels in treated tumors and that this down-regulation is likely mediated through IFN-\( \gamma \) produced by IL-12 stimulation. This study also showed that IL-12 treatment was associated with a marked decrease in matrix metalloproteinase 9, an enzyme important in endothelial cell migration and angiogenesis, and an increase in the expression of tissue inhibitor of metalloproteinase 1, a known inhibitor of matrix metalloproteinase 9 (52). In a recent study by Gee et al. (12), IL-12
was shown to inhibit the growth of K1735 melanoma tumors in C3H/HeN mice in an IFN-γ-dependent manner. Using in vivo Matrigel assays, IFN-γ blockade was shown to abrogate IL-12-induced inhibition of neovascularization in K1735 implants. Interestingly, IL-12-induced IFN-γ was also shown to produce direct cellular apoptosis independent of the hypoxia-related apoptosis associated with angiogenesis. These studies suggest that IFN-γ produced intratumorally in response to IL-12 treatment is capable of inhibiting tumor growth through two independent mechanisms: (a) directly through induction of tumor cell apoptosis; and (b) through inhibition of angiogenesis producing cellular hypoxia and hypoxia-related apoptosis.

In conclusion, our studies show that in vivo transduction of growing murine hepatocellular tumors with AdVmIL-12 leads to profound tumor growth regression. This AdVmIL-12-mediated regression could not be abrogated in models designed to inhibit NK T-cell function and other cytokotoxoid immune mechanisms. AdVmIL-12 treatment was associated with a rapid infiltration of lymphoid cells and clearly demonstrable inhibition of tumor-associated neovascularization. These data clearly demonstrate that NK T cells are not absolutely required for IL-12-mediated antitumor responses and suggest that mechanisms independent of direct cellular cytotoxicity initiate the effects of IL-12 demonstrated in murine HCC models.

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