

Antitumor and Antimetastatic Activity of Interleukin 12 against Murine Tumors

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Summary

It has recently been demonstrated that in vivo administration of murine interleukin 12 (IL-12) to mice results in augmentation of cytotoxic natural killer (NK)/lymphocyte-activated killer cell activity, enhancement of cytolytic T cell generation, and induction of interferon γ secretion. In this study, the in vivo activity of murine IL-12 against a number of murine tumors has been evaluated. Experimental pulmonary metastases or subcutaneous growth of the B16F10 melanoma were markedly reduced in mice treated intraperitoneally with IL-12, resulting in an increase in survival time. The therapeutic effectiveness of IL-12 was dose dependent and treatment of subcutaneous tumors could be initiated up to 14 d after injection of tumor cells. Likewise, established experimental hepatic metastases and established subcutaneous M5076 reticulum cell sarcoma and Renca renal cell adenocarcinoma tumors were effectively treated by IL-12 at doses which resulted in no gross toxicity. Local peritumoral injection of IL-12 into established subcutaneous Renca tumors resulted in regression and complete disappearance of these tumors. IL-12 was as effective in NK cell-deficient beige mice or in mice depleted of NK cell activity by treatment with antisialoside GM1, suggesting that NK cells are not the primary cell type mediating the antitumor effects of this cytokine. However, the efficacy of IL-12 was greatly reduced in nude mice suggesting the involvement of T cells. Furthermore, depletion of CD8⁺ but not CD4⁺ T cells significantly reduced the efficacy of IL-12. These results demonstrate that IL-12 has potent in vivo antitumor and antimetastatic effects against murine tumors and demonstrate as well the critical role of CD8⁺ T cells in mediating the antitumor effects against subcutaneous tumors.

Human IL-12 is a disulfide-bonded heterodimeric cytokine consisting of a 40- and a 35-kD subunit (1, 2). The genes for this cytokine have been cloned (3, 4) and purified recombinant protein has been produced. A number of biological properties of human IL-12 have been evaluated in vitro. Among its properties are the ability to act as a NK cell and T cell growth factor (5-7), to enhance NK/LAK cell cytolytic activity (1, 7-9), to augment cytolytic T cell responses (9), and to induce secretion of cytokines, particularly IFN- γ , from T and NK cells (1, 10). Since both T and NK cells have been implicated as antitumor effector cells (11) and IFN- γ has been shown to have antitumor activity in animals (12, 13), IL-12 has the potential to be used as an immunomodulatory cytokine in the therapy of malignancies.

The ability to test the in vivo activities of IL-12 have been limited since human IL-12 is inactive on murine cells, but recently the genes for murine IL-12 have been cloned (14). Injection of mice with recombinant murine IL-12 augments

NK activity, enhances allogeneic cytolytic T cell responses, and induces secretion of IFN- γ , thus confirming the previously described in vitro activities of IL-12 (Gately, M. K., R. R. Warriar, S. Honasoge, D. A. Faherty, S. E. Connaughton, T. D. Anderson, U. Sarmiento, B. R. Hubbard, and M. Murphy, manuscript submitted for publication). Based on these in vivo results, the antitumor and antimetastatic activities of murine IL-12 against a number of murine malignancies have been evaluated. We demonstrate in this study that systemic administration of IL-12 can inhibit the growth of both established subcutaneous tumors and experimental pulmonary or hepatic metastases, and that local peritumoral injections of IL-12 can result in regression of established subcutaneous tumors. Based on results obtained using mice deficient in lymphocyte subsets and antibody depletion experiments, the antitumor efficacy of IL-12 is mediated primarily through CD8⁺ T cells.

Materials and Methods

Mice. C57BL/6, C57BL/6 *bg/bg* beige, C57BL/6 *bg/+* heterozygous beige control and BALB/c mice, 6–8 weeks of age, were purchased from The Jackson Laboratories, (Bar Harbor, ME). BALB/c nude mice were obtained from Harlan-Sprague Dawley, Inc. (Madison, WI). Mice were routinely screened and found to be free of mycoplasma and selected murine viruses.

Tumor Cell Lines. B16F10 malignant melanoma cells (15) and Renca renal cell adenocarcinoma cells (16), obtained from Dr. R. Wiltout (Biological Response Modifiers Program, Frederick, MD), were maintained *in vitro* in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 25 mM NaHCO₃, and 60 μ g/ml L-glutamine. M5076, a reticulum cell sarcoma (12, 17), was maintained as a stationary suspension culture in RPMI 1640 supplemented with 17% equine serum (Hyclone Laboratories, Inc., Logan, UT), 1 mM sodium pyruvate, 50 μ g/ml gentamicin, glutamine, penicillin, and streptomycin. All reagents for cell culture were purchased from GIBCO BRL (Gaithersburg, MD) unless otherwise designated. All cell lines were found to be free of mycoplasma and viruses as above.

Recombinant Murine IL-12. Murine rIL-12 was expressed in CHO cells that had been stably transfected with the IL-12 p40 and p35 cDNAs and was purified as previously described (Gately, M. K., et al., manuscript submitted for publication). SDS-PAGE analysis of the purified IL-12 indicated it to be $\geq 95\%$ pure with a small amount of contaminating IL-12 p40 monomer. Monomeric IL-12 p40 does not bind to the IL-12 receptor (18) and lacks biological activity (3, 4). Contamination by endotoxin, as assessed by the *Limulus* ameocyte assay, was < 5 EU/mg IL-12. The sp act of purified murine rIL-12, as determined by its ability to cause proliferation of human PHA blasts (18), was $\sim 7 \times 10^6$ U/mg protein. For administration to mice, rIL-12 was diluted in PBS containing 100 μ g/ml mouse serum albumin (Miles Scientific, Naperville, IL or Sigma Chemical Co., St. Louis, MO).

Tumor Experiments. Exponentially growing tumor cells were harvested by brief trypsinization (B16F10 and Renca), washed, and injected subcutaneously or intravenously into groups of 10 mice on day 0, and intraperitoneal treatment with various doses of IL-12 once per day five times per week was initiated between days 1 and 28, depending on the experiment. Specific protocols are presented in the legends of individual experiments. The diameters of subcutaneous tumors were measured twice weekly with calipers, and volume was calculated by the formula: Vol = (longest diameter) \times (shortest diameter)² (Gately, M. K., et al., manuscript submitted for publication). For metastasis experiments, approximately 3 wk after intravenous injection of tumor cells, mice were killed and the number of metastases enumerated as previously described (15). Statistical evaluation of the data was performed using the non-parametric one-tailed Mann-Whitney U test (17). Experiments were repeated two to eight times, and data from representative experiments are presented.

Antibody Depletion Experiments. Antisialo GM1 (anti-ASGM1) was purchased from Biochemical Diagnostics, Inc. (Edgewood, NY). Mice were injected intraperitoneally with 0.4 ml of 1:5 dilution of anti-ASGM1 or normal rabbit serum on the days indicated. Under these conditions of treatment with antisialo GM1 splenic NK activity, is reduced to undetectable levels (17).

Anti-CD4 (clone GK1.5, rat IgG2b) (19) and anti-CD8 (clone 2.43, rat IgG2b) (20) hybridomas were purchased from the American Type Culture Collection (Rockville, MD). Ascites were pre-

pared in pristane-treated nude mice and Ig purified by the procedure of Reik et al. (21). Mice were injected intraperitoneally with 1 mg of Ig on the days indicated. Under these conditions, antibody treatment depleted $> 95\%$ of the appropriate cell population in the spleen.

Results

We initially evaluated the effect of IL-12 on experimental pulmonary metastases of the B16F10 melanoma. After intravenous injection with tumor cells on day 0, C57BL/6 mice were treated intraperitoneally beginning on day 1 with varying doses of IL-12 five times per week for 3 wk. At the end of the treatment period, there was a dose-dependent inhibition of experimental metastases in the IL-12-treated mice (Fig. 1A). In the group receiving 1 μ g per injection, the median number of metastases was reduced to 41 compared with 200 in the diluent-treated mice ($p < 0.005$). To determine if treatment could be initiated at a time when metastases were al-

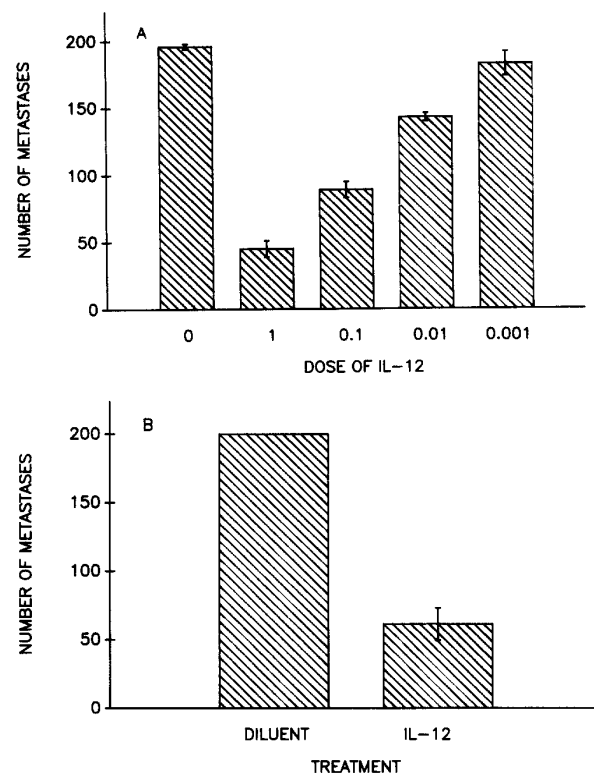


Figure 1. Inhibition of B16F10 metastases by IL-12. (A) Mice were injected intravenously with 2×10^5 B16F10 cells on day 0 and treatment with diluent or varying doses of IL-12 per injection was initiated intraperitoneally on day 1. Mice were treated five times per week until animals were killed on day 22. (B) Treatment with 1 μ g per injection of IL-12 five times per week was initiated on day 7 and mice were killed on day 22. Data are represented as mean \pm SE of 10 mice per group.

ready firmly established, mice were similarly injected intravenously with tumor cells on day 0, but the initiation of treatment was delayed until day 7. As shown in Fig. 1 B, under these experimental conditions, treatment with 1 μg of IL-12 also significantly reduced the number of experimental

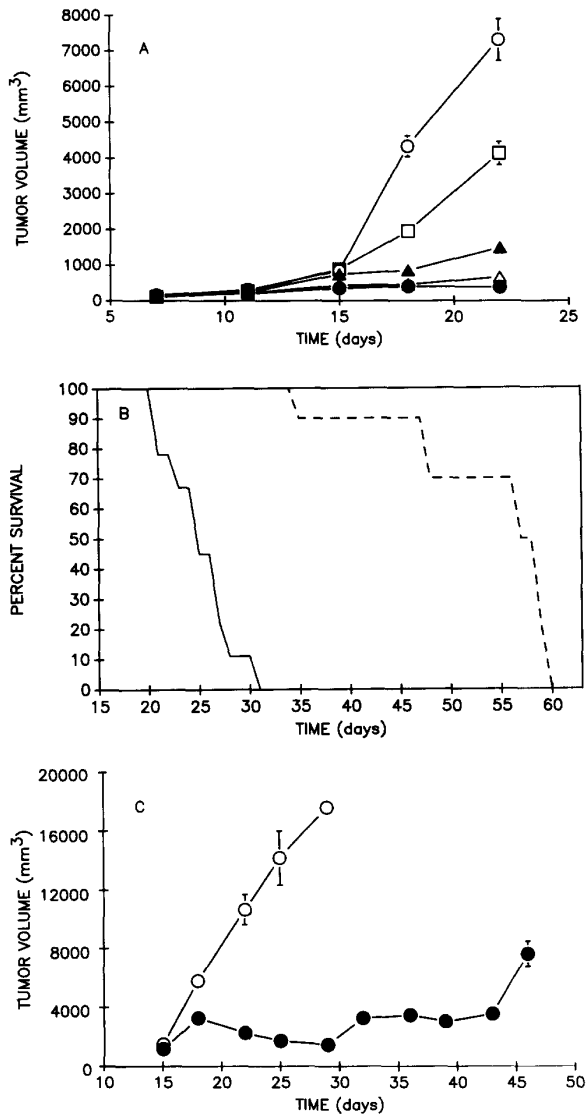


Figure 2. Inhibition of subcutaneous growth of B16F10 tumors. (A) Mice were injected subcutaneously with 10^6 B16F10 cells on day 0 and intraperitoneal treatment with diluent (○—○) or 5 μg (●—●), 1 μg (△—△), 0.1 μg (▲—▲), or 0.01 μg (□—□) of IL-12 was initiated on day 7. Mice were treated five times per week until animals were killed on day 22. (B) Survival of mice treated intraperitoneally with diluent (solid line) or 1 μg of IL-12 (dashed line) per injection five times per week through day 50. (C) Treatment of mice was initiated on day 14. Mice were treated intraperitoneally with diluent (○—○) or 1 μg of IL-12 (●—●) per injection five times per week for 3 wk. Data are represented as mean \pm SE of 10 mice per group.

metastases ($p < 0.0005$). It is clear from these results that IL-12 has potent antitumor activity in this experimental metastasis model and that treatment of animals with IL-12 can be initiated after metastases have already been established.

A series of experiments was then performed to determine if IL-12 also has activity against subcutaneously growing B16F10 tumors. Initially, mice were injected subcutaneously with 10^6 tumor cells on day 0, and treatment with varying amounts of IL-12 was initiated on day 7 when small tumors were present in the animals. As previously demonstrated with experimental metastases, there was a dose-dependent inhibition of tumor growth with maximal effects observed at a dose of 1 μg per injection (Fig. 2 A). At all doses tested, no gross toxicity was evident and all animals survived the therapy, although at the 5 μg dose some lethargy was observed. To determine if IL-12 could increase the survival of B16F10 tumor-bearing mice and to test if the effect of prolonged IL-12 treatment on tumor growth, tumor-bearing mice were treated with 1 μg per injection of IL-12 five times per week until day 50. As seen in Fig. 2 B, there was a large increase in survival time of IL-12-treated mice compared with

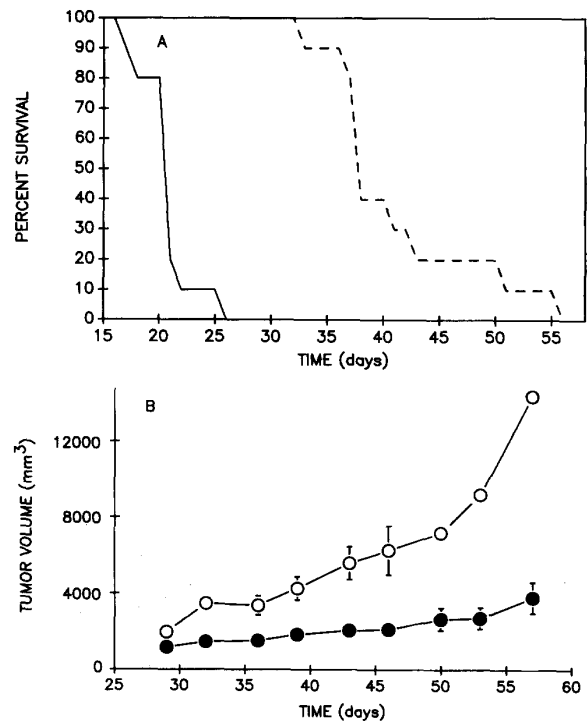


Figure 3. Inhibition of M5076 experimental hepatic metastases and subcutaneous tumor growth by IL-12. (A) Survival of mice injected intravenously on day 0 with 10^5 M5076 tumor cell and treated intraperitoneally with diluent (solid line) or 1 μg of IL-12 (dashed line) per injection five times per week for 3 wk was initiated on day 1. (B) Mice were injected subcutaneously with 10^6 M5076 tumor cells on day 0 and intraperitoneal treatment with diluent (○—○) or 1 μg of IL-12 (●—●) per injection was initiated on day 28. Animals were treated five times per week for 4 wk. Data represented as mean \pm SE of 10 mice per group.

diluent-treated control mice. Although tumor growth was suppressed for a prolonged time by IL-12 treatment, no "cures" were obtained, and tumors eventually grew, resulting in death of the animals. To evaluate the effect of IL-12 against larger B16F10 tumors, treatment with 1 μg of IL-12 per injection was delayed until day 14. As seen in Fig. 2 C, tumors grew progressively in control animals through day 29, at which point all animals had died. In contrast, the growth of tumors in mice treated with IL-12 was greatly diminished. These results demonstrate that IL-12 has marked antitumor activity against subcutaneous B16F10 tumors.

Since antitumor activity of IL-12 had only been demonstrated against the B16F10 melanoma, the antitumor efficacy of IL-12 was next evaluated against additional tumors. After intravenous injection of M5076 reticulum cell sarcoma, experimental hepatic metastases are formed (17). As seen in Fig. 3 A, diluent-treated mice have a median survival of 21 d with all animals dead by day 26, but treatment of mice with IL-12 results in an increase in median survival time to 38 d. After subcutaneous injection of M5076 tumor cells, tumors develop much more slowly than in mice injected with B16F10 melanoma cells. Thus, intraperitoneal treatment of mice with IL-12 was begun 4 wk after implantation of tumor cells. Under these conditions, IL-12 markedly reduced tumor growth (Fig. 3 B). In these IL-12-treated animals, spontaneous hepatic metastases are also greatly reduced (Brunda, M. J., and T. D. Anderson, unpublished observation). Therefore, the antitumor activity of IL-12 is evident against both subcutaneous tumor growth and experimental hepatic metastases of the M5076 reticulum cell sarcoma.

To evaluate further the antitumor activity of IL-12, mice were injected subcutaneously with the Renca renal cell adenocarcinoma and treated with IL-12. The Renca renal cell adenocarcinoma is a murine tumor of spontaneous origin in BALB/c mice that mimics histologically renal cell carcinoma in humans (16) and is sensitive to various cytokines in vivo (16, 22). Beginning on day 14, intraperitoneal treatment of mice with IL-12 resulted in a dramatic inhibition of tumor growth (Fig. 4 A), although complete regression of tumors was not observed. Since it has previously been reported that regression of the Renca tumor occurs after transfection of IL-4 into this tumor cell (22), mice bearing day 14 subcutaneous Renca tumors were injected peritumorally with 1 μg of IL-12. As seen in Fig. 4 B, tumors were markedly inhibited and, in fact, tumor regression occurred with no tumor detectable in 70% of treated mice after 60 d. Upon rechallenge with Renca cells alone, tumor growth is greatly inhibited in these survivors (data not shown). Thus, IL-12 has pronounced antitumor activity against the Renca renal cell adenocarcinoma and can, under certain conditions, result in regression of established tumors.

Since IL-12 has been demonstrated to have effects on both NK and T cells (1–10, 14, and Gately, M. K., et al., manuscript submitted for publication), the antitumor activity of IL-12 might be mediated through either of these cell types. To determine if the antitumor efficacy of IL-12 was primarily mediated through NK cells, its activity was evaluated against

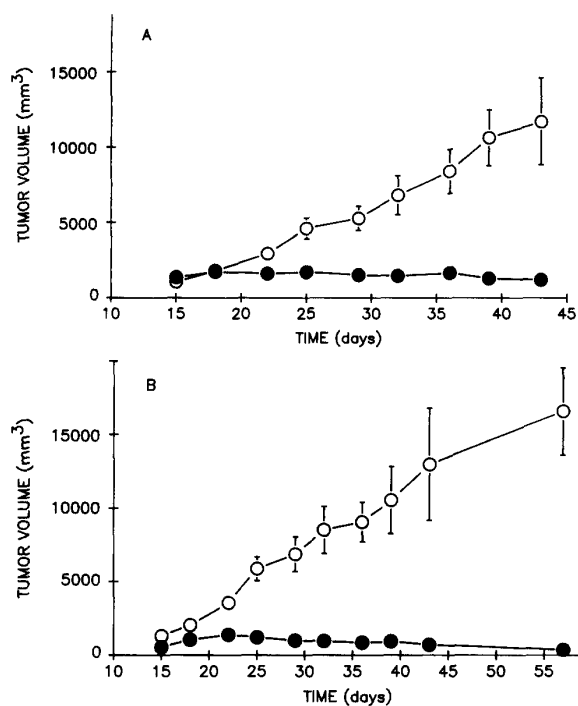


Figure 4. Inhibition of Renca tumors by IL-12. Mice were injected subcutaneously on day 0 with 10^6 Renca tumor cells and treatment with diluent (○—○) or 1 μg of IL-12 (●—●) per injection five times per week for 4 wk was initiated on day 14. (A) Treatment by intraperitoneal route. (B) Treatment by peritumoral route. Data represented as mean \pm SE of 10 mice per group.

B16F10 tumors in NK cell deficient *bg/bg* beige (23). Treatment of either *bg/bg* beige or phenotypically normal *bg/+* heterozygotes with 1 μg per injection of IL-12 resulted in a comparable degree of tumor inhibition (Fig. 5). To confirm and extend these results, Renca tumor-bearing BALB/c mice were depleted of NK cells using anti-ASGM1 and treated with IL-12. No loss of IL-12-induced antitumor efficacy was observed in these NK cell-depleted mice (Fig. 6). Taken together, these results strongly suggest that the antitumor efficacy of IL-12 is not mediated through NK cells.

To evaluate the potential role of T cells on IL-12-induced antitumor efficacy, the antitumor effect of IL-12 was tested against B16F10 tumors in nude mice (Fig. 7 A). Although there was a slight delay in tumor growth, the effect of IL-12 was markedly diminished compared with that observed in euthymic mice. The greatly reduced activity of IL-12 was also obtained in BALB/c nude mice bearing syngeneic Renca tumors (Fig. 7 B). To evaluate further the requirement for T cells in mediating IL-12-induced antitumor efficacy and to determine the role of T cell subsets, Renca tumor-bearing mice were treated with anti-CD4 or anti-CD8 antibodies. As seen in Fig. 8, the activity of IL-12 is not affected in mice depleted of CD4⁺ T cells but substantially, although not

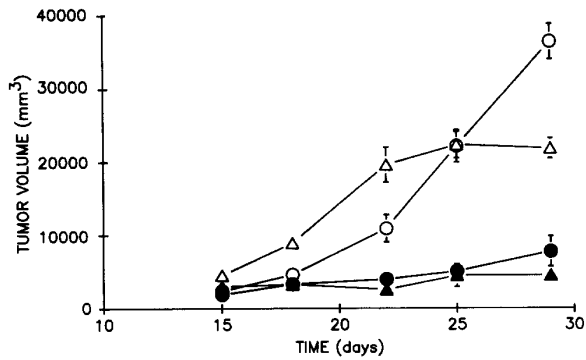


Figure 5. Antitumor effects of IL-12 in NK cell-deficient beige mice. Animals were injected subcutaneously with 10^6 B16F10 tumor cells on day 0 and intraperitoneal treatment with diluent (open symbols) or $1 \mu\text{g}$ of IL-12 (filled symbols) per injection five times per week until the end of the experiment was initiated on day 14 in *bg/+* heterozygous mice (O/●) or *bg/bg* beige mice (Δ/\blacktriangle). Data are represented as mean \pm SE of 10 mice per group.

completely, lost in mice treated with anti-CD8. These results demonstrate that CD8⁺ T cells are critical for mediating the antitumor effects of IL-12.

Discussion

Using purified recombinant murine IL-12, we have demonstrated that this cytokine has potent antitumor and antimetastatic activity against a number of murine tumors of various histological types. Therapeutic intervention by systemic administration of IL-12 can be initiated when tumors or metastases are well established, up to day 28 after injection.

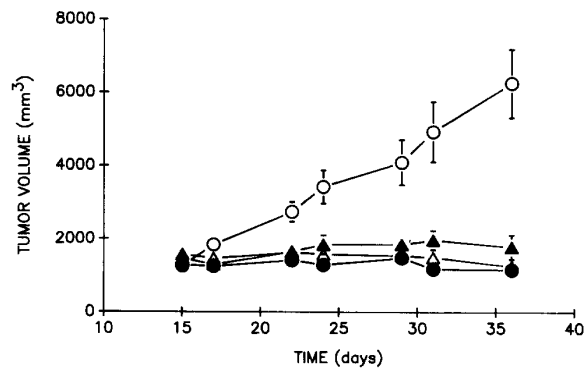


Figure 6. Antitumor effects of IL-12 in mice treated with anti-ASGM1. Animals were injected subcutaneously with 10^6 Rencu tumor cells on day 0 and intraperitoneal treatment with diluent (O—O) or IL-12 (●—●) was initiated on day 14 and continued five times per week for 4 wk. Other groups were treated with IL-12 beginning on day 14 and were injected intraperitoneally with either NRS (Δ — Δ) or anti-ASGM1 (\blacktriangle — \blacktriangle) on days 15, 17, 21, 24, 28, and 31. Data are represented as mean \pm SE of 10 mice per group.

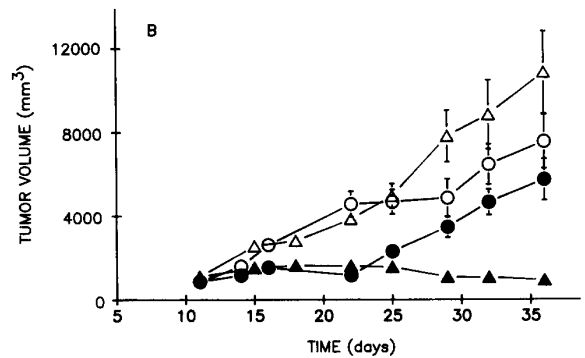
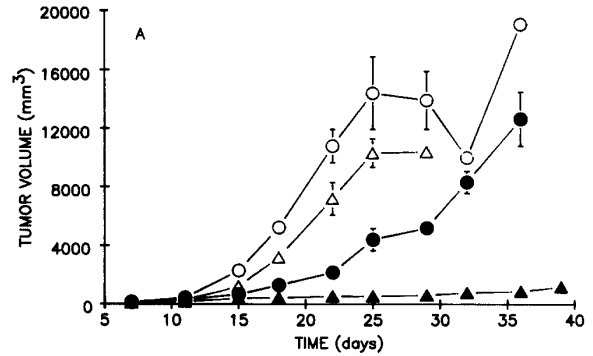


Figure 7. Reduced efficacy of IL-12 in tumor-bearing nude mice. Mice were treated intraperitoneally five times per week for 4 wk with diluent (open symbols) or $1 \mu\text{g}$ IL-12 (closed symbols) beginning on day 7. Data are represented as mean \pm SE of 10 mice per group. (A) B6 (Δ/\blacktriangle) or BALB/c nude mice (O/●) were injected subcutaneously with 10^6 B16F10 tumor cells on day 0. (B) BALB/c (Δ/\blacktriangle) or BALB/c nude mice (O/●) were injected subcutaneously with 10^6 Rencu tumor cells on day 0.

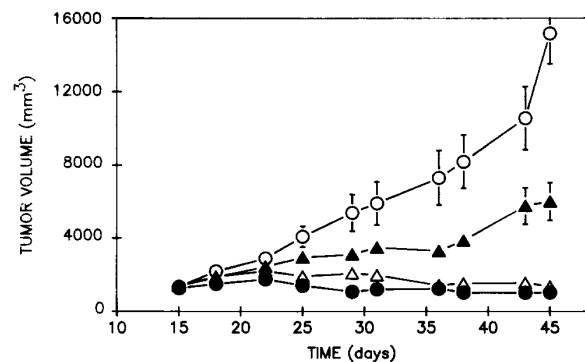


Figure 8. Effect of depletion of CD4⁺ or CD8⁺ T cells on antitumor efficacy of IL-12. Mice were injected subcutaneously with 10^6 Rencu tumor cells on day 0 and intraperitoneal treatment five times per week until the end of the experiment with diluent (O—O) or $1 \mu\text{g}$ IL-12 (●—●) was initiated on day 14. Other groups of mice were injected with IL-12 and either anti-CD4 (Δ — Δ) or anti-CD8 (\blacktriangle — \blacktriangle) on days 14, 21, and 28. Data are represented as mean \pm SE of 10 mice per group.

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