Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2219–2223, March 1995
Immunology

Influence of interleukin 12 on p53 peptide vaccination against established Meth A sarcoma

(tumor immunology/cancer vaccines)

YUJI NOGUCHI*, ELIZABETH C. RICHARDS*, YAO-TSENG CHEN*†, AND LLOYD J. OLD*

*Ludwig Institute for Cancer Research, New York Unit at Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021; and †Department of Pathology, New York Hospital–Cornell Medical Center, 1300 York Avenue, New York, NY 10021

Contributed by Lloyd J. Old, November 28, 1994

ABSTRACT **BALB/c murine sarcoma Meth A is known to have three missense point mutations in p53. We previously reported that a nonamer peptide containing the codon 234 mutational product (designated 234CM) elicited 234CM-specific cytotoxic T cells and that immunization with 234CM in adjuvant before tumor challenge inhibited Meth A growth. Because interleukin 12 (IL-12) has been shown to have antitumor activity against established tumors and immunomodulatory activities, we analyzed its effect on p53 peptide immunization and Meth A growth. Multiple injections of IL-12 alone (4 times a week for 2 weeks) caused regression of established Meth A sarcoma, and this effect was dose dependent. IL-12 treatment prior to Meth A challenge had little or no antitumor activity. To evaluate the effect of IL-12 on the generation of 234CM-specific cytotoxic T lymphocytes, spleen cells from BALB/c mice immunized with 234CM in adjuvant and injected with various doses of IL-12 were sensitized with 234CM *in vitro*. Multiple injections of 1 ng of IL-12 induced the highest cytotoxicity against target cells pulsed with 234CM. Higher doses of IL-12 suppressed 234CM-specific cytotoxic T-cell generation. Mice immunized with 234CM in QS-21 adjuvant and treated with 1 ng of IL-12 rejected established Meth A sarcoma. Mice comparably treated with 1 ng of IL-12 but immunized with 234CW peptide (the wild-type counterpart to 234CM) in QS-21 or with QS-21 alone showed progressive tumor growth.**

Striking progress has been made in the structural identification of the peptide targets of cytotoxic T lymphocytes (CTLs) on mouse and human tumors (1), raising hopes that effective vaccines can be constructed by using these cancer products. In experimental systems, immunization with tumor antigens elicits heightened resistance to tumor challenge but generally has little or no effect on established tumors. Two notable exceptions to this rule are the results of immunization with Renca renal cell carcinoma transfected with interleukin 4 (IL-4; ref. 2) and with the mutated connexin 37 peptide in the Lewis lung cancer (3). In the case of BALB/c murine Meth A sarcoma, vaccination with a mutated p53 peptide (234CM) in incomplete Freund's adjuvant (IFA) increases resistance to Meth A challenge (4) but does not significantly influence the growth of established Meth A tumors. To explore how to make tumor peptide vaccination more effective, we have begun to analyze different adjuvants, such as QS-21 (5, 6), and cytokines such as IL-12 (7, 8). IL-12 is a heterodimeric cytokine that has been shown to have strong antitumor activity by itself (9, 10), mediated in large part by its ability to induce interferon γ (IFN-γ) (10). IL-12 is also known to play a critical role in the differentiation of T helper 0 (T_H0) to T_H1 and T_H2 to T_H0 cells *in vitro* (11, 12). In the present study, we report that IL-12 enhances the generation of 234CM-specific CTLs and that vaccines containing 234CM peptide in QS-21 cause regression of established Meth A tumors in mice treated with IL-12 at a dose that lacks antitumor activity by itself.

MATERIALS AND METHODS

Mice. BALB/c female mice were obtained from the animal facility at Memorial Sloan–Kettering Cancer Center or purchased from The Jackson Laboratory.

Tumor Cells. Meth A is a transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passaged as an ascitic tumor (13). P1-HTR is a cell line derived from P815 mastocytoma of DBA/2 origin (14).

Peptides. 234CM (KYICNSSCM) is a nonamer containing isoleucine at position 3 that results from a point mutation in codon 234 of Meth A p53. 234CW (KYMCNSSCM) is the wild-type counterpart to 234CM. Both peptides were purchased from Appligene (Pleasanton, CA), and the purity of the two peptides was confirmed by mass spectrometry.

Adjuvants. IFA was purchased from Sigma. QS-21 was kindly provided by Cambridge Biotech and dissolved in Dulbecco's phosphate-buffered saline (D-PBS; Life Technologies) at a concentration of 1 mg/ml. A 10 μg dose of QS-21 in 200 μl was used for each immunization. Half of the mice injected intradermally with this dose develop small ulcers at the injection site. For details regarding QS-21, see ref. 5.

Recombinant Murine IL-12. Recombinant murine IL-12 was kindly provided by M. K. Gately (Hoffmann–La Roche). The recombinant IL-12 was diluted with PBS containing 1% syngeneic mouse serum and injected intraperitoneally in a volume of 0.2 ml. For details regarding recombinant murine IL-12, see refs. 7 and 15.

Monoclonal Antibodies (mAbs). Anti-L3T4 (CD4) mAb and anti-Lyt-2.2 (CD8) were kindly provided by F. Fitch (University of Chicago) and U. Hammerling (Memorial Sloan–Kettering Cancer Center), respectively. For *in vivo* T-cell depletion, mice were injected intravenously with 50 μl of ascitic fluid diluted in 200 μl of RPMI-1640 culture medium.

Generation and Analysis of Peptide-Specific CTLs. For details of the ^{51}Cr-release assay used to evaluate CTL activity, see ref. 4.

RESULTS

Antitumor Effect of IL-12 on Meth A Sarcoma. We initially evaluated the antitumor effect and dose dependency of IL-12 on established Meth A sarcoma. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells on day 0, and 7 days later treatment was started with different doses of IL-12 injected intraperitoneally (4 times a week for 2 weeks). Fig. 1 shows that the antitumor effect of IL-12 was dose dependent

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CTL, cytotoxic T lymphocyte; IFN-γ, interferon γ; IFA, incomplete Freund's adjuvant; IL-*n*, interleukin *n*; T_Hn cells, T helper *n* cells; mAb, monoclonal antibody.

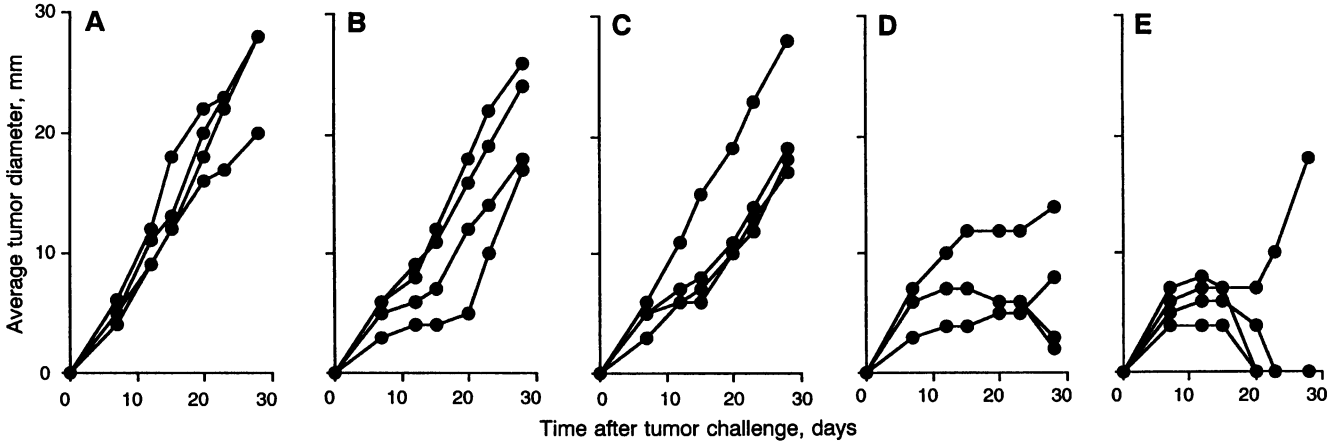

FIG. 1. Antitumor effect of IL-12 on established Meth A sarcoma. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells on day 0 and were injected intraperitoneally (four mice per group) with 0 ng (*A*), 1 ng (*B*), 10 ng (*C*), 100 ng (*D*), or 500 ng (*E*) of IL-12 (4 times a week for 2 weeks). Injections of IL-12 were initiated on day 7.

and that regression of Meth A sarcoma was seen in mice treated with the higher doses of IL-12. This antitumor effect was abolished by treatment on day 7 with anti-CD8 mAb but not anti-CD4 mAb. IL-12 was not effective when injected before Meth A challenge (Fig. 2*A*) and showed only a moderate antitumor effect when treatment was initiated at the time of tumor challenge (Fig. 2*B*).

Effect of IL-12 on the Generation of Peptide Specific CTLs. We next evaluated the effect of IL-12 on the generation of 234CM-specific CTLs. Mice were vaccinated intradermally with 100 μg of 234CM in IFA or QS-21 on day 0 and day 7. Different doses of IL-12 were injected intraperitoneally (4 times a week for 2 weeks) starting on day 0. On day 14, spleen cells from immunized mice were sensitized with syngeneic spleen cells pulsed with 234CM. CTL activity of responding cells was evaluated by a ^{51}Cr-release assay. Fig. 3 shows that 1 ng of IL-12 augmented CTL generation in mice immunized with 234CM in IFA. This effect of IL-12 on CTL generation was consistently seen in mice immunized with 234CM in IFA but not in mice immunized with 234CM in QS-21. Higher doses of IL-12 had a suppressive effect. No CTLs were elicited after immunization with 100 μg of 234CW (the wild-type counterpart to 234CM) in IFA or QS-21, and treatment with IL-12 did not influence this response.

Effect of Peptide Immunization and IL-12 on Established Meth A Sarcoma. We next asked whether the combination of peptide immunization and low-dose IL-12 treatment could suppress the growth of established tumors. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells on day 0. Mice were immunized intradermally at a distant site with 100 μg of 234CM in QS-21 on day 7 and day 14 and injected intraperitoneally with 1 ng of IL-12 (4 times a week for 2 weeks) starting on day 7. Fig. 4 shows that the growth of Meth A was suppressed in IL-12-treated mice immunized with 234CM in QS-21 but not in IL-12-treated mice immunized with 234CW in QS-21 or injected with either QS-21 or 234CM alone. As indicated by antibody depletion tests initiated on day 7, this tumor suppression by peptide vaccination and IL-12 was mediated by both $CD4^+$ and $CD8^+$ T cells (Fig. 5). When antibody was given on day 15, however, only anti-CD8 mAb abolished the protective effect of the vaccine. QS-21 is a more effective adjuvant than IFA in inducing this 234CM-specific regression of Meth A in IL-12-treated mice, even though immunization with 234CM in IFA generated higher 234CM-specific CTL activity than QS-21 (see above). In the absence of IL-12 treatment, no tumor regression was seen in mice vaccinated with 234CM in QS-21 or IFA.

DISCUSSION

As shown in this study, regression of established Meth A sarcoma transplants can be induced by high doses of IL-12 alone or by vaccination with 234CM in adjuvant and low doses of IL-12. One mechanism to account for this low-dose effect

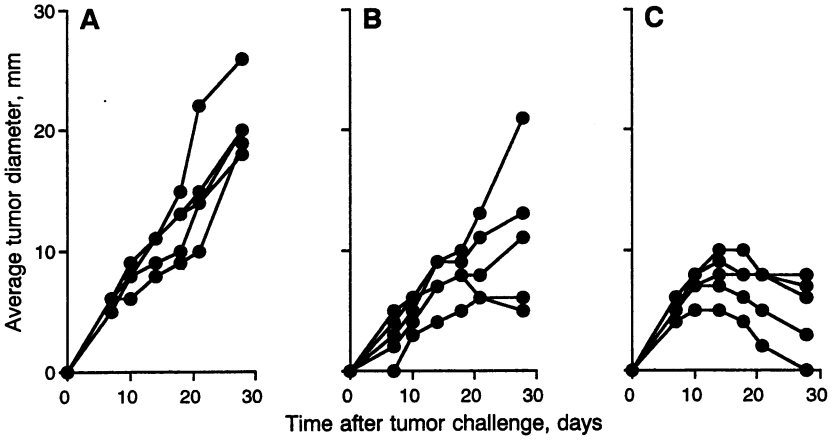

FIG. 2. Antitumor effect of IL-12 in relation to time of tumor challenge. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells on day 0. Mice were injected intraperitoneally (five mice per group) with 100 ng of IL-12 (4 times a week for 2 weeks). Injection with IL-12 was initiated 14 days before tumor challenge (*A*), at the time of tumor challenge (*B*), or 7 days after tumor challenge (*C*).

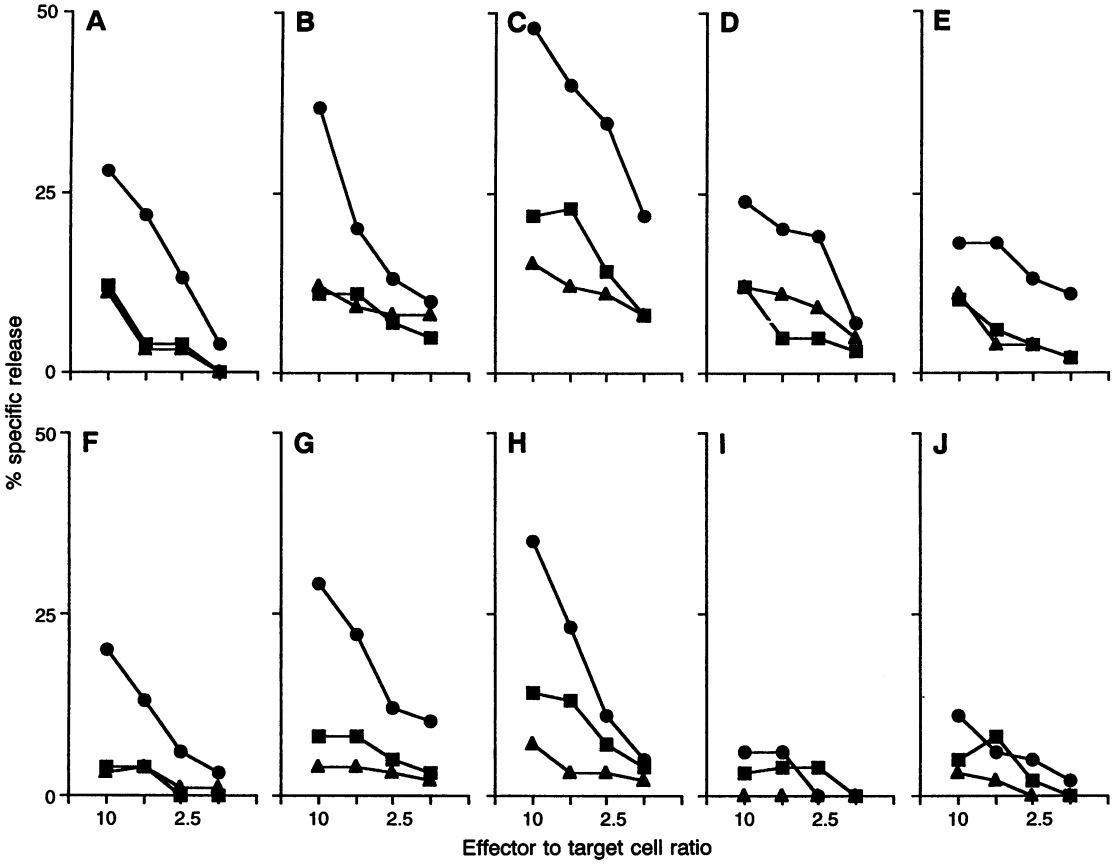

FIG. 3. Effect of IL-12 on the generation of 234CM-specific CTLs. BALB/c female mice were immunized intradermally with 100 μg of 234CM in IFA (*A–E*) or QS-21 (*F–J*) on day 0 and day 7 and injected with 0 ng (*A* and *F*), 0.1 ng (*B* and *G*), 1 ng (*C* and *H*), 10 ng (*D* and *I*), or 100 ng (*E* and *J*) of IL-12 (4 times a week for 2 weeks) starting on day 0. Spleen cells from individual mice were harvested on day 14, sensitized with 234CM-pulsed spleen cells, and tested in the ^{51}Cr-release assay for CTL activity by using P1-HTR target cells pulsed with 234CM (●) or 234CW (■) or P1-HTR cells that had not been pulsed with a peptide (▲).

of IL-12 comes from our finding that such low doses can facilitate the generation of 234CM-specific CTLs. Higher doses of IL-12 actually suppress 234CM-specific CTL generation. This phenomenon of low-dose augmentation and high-dose suppression of CTL generation by IL-12 has been seen in a virus system, where lymphocytic choriomeningitis virus-specific CTL activity was inhibited by the treatment with high doses of IL-12 (16). Despite the suppressive effect of high doses of IL-12 on CTL generation, these levels of IL-12 have potent antitumor activity. In the case of Meth A, the antitumor effect of IL-12 alone is most striking with established tumors; treatment with IL-12 before or at the time of tumor challenge has little or no effect. Thus, it appears that immune responses to Meth A antigens must be under way for IL-12 to exert its antitumor effect, and this seems to be the case for the low-dose effect of IL-12 as well; 1 ng of IL-12 does not augment the antitumor effect of 234CM in adjuvant when vaccination is carried out before Meth A challenge. With regard to IL-12

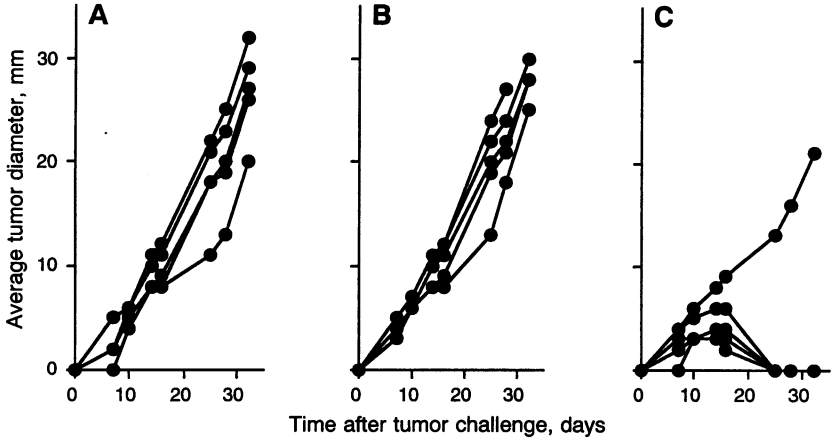

FIG. 4. Suppression of growth of established Meth A by peptide vaccination in combination with low-dose IL-12 treatment. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells on day 0 and vaccinated intradermally (five mice per group) at a distant site with QS-21 alone (*A*), 100 μg of 234CW in QS-21 (*B*), or 100 μg of 234CM in QS-21 (*C*) on day 7 and day 14. Mice were injected with 1 ng of IL-12 (4 times a week for 2 weeks) starting on day 7.

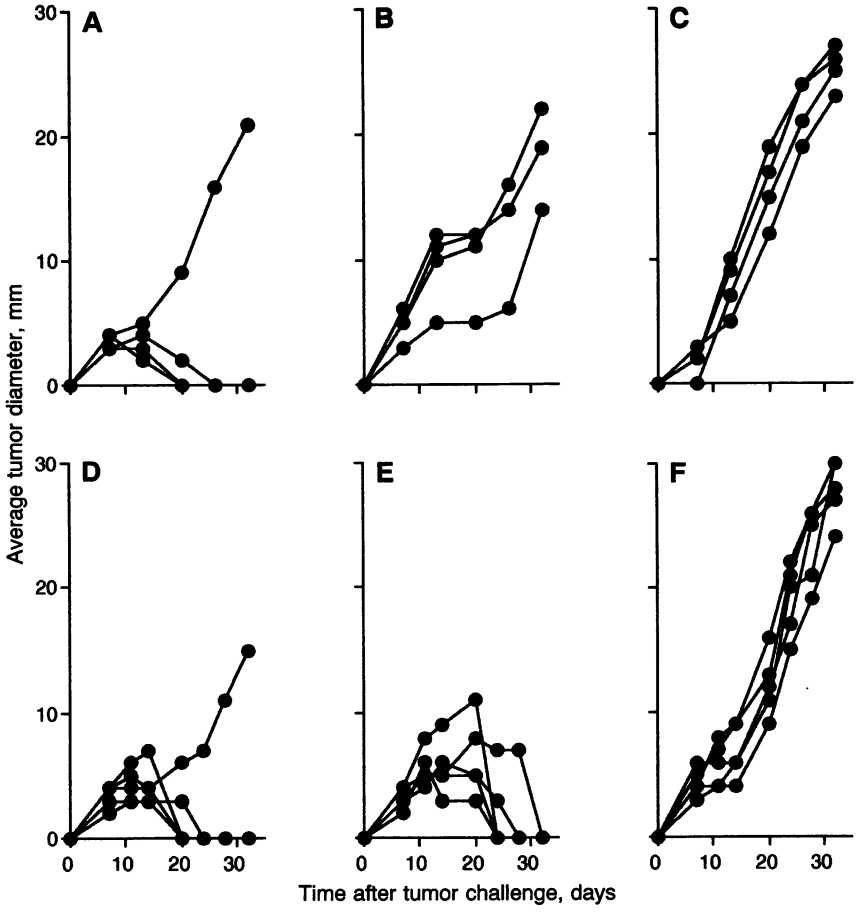

FIG. 5. $CD4^+$ and $CD8^+$ T-cell involvement in regression of Meth A by peptide vaccination and low-dose IL-12 treatment. BALB/c female mice were injected intradermally with 5×10^4 Meth A cells. Mice were vaccinated intradermally at a distant site with 100 μg of 234CM in QS-21 on day 7 and day 14 and injected with 1 ng of IL-12 (4 times a week for 2 weeks) starting on day 7. Mice were injected intravenously on day 7 (*A*, *B*, and *C*) or day 15 (*D*, *E*, and *F*) with no mAb (*A* and *D*), anti-CD4 mAb (*B* and *E*), or anti-CD8 mAb (*C* and *F*).

action, IL-12 is a strong inducer of IFN-γ (15), and most, if not all, of the antitumor activity of IL-12 appears to be mediated by IFN-γ, as indicated by neutralization studies with anti-IFN-γ antibodies *in vivo* (10). Antibody neutralization studies have also shown that IFN-γ is a critical mediator in the regression of established Meth A sarcoma by lipopolysaccharide (17). Experiments with Meth A cells transfected with dominant negative IFN-γ receptors, thereby incapacitating the cellular response to the IFN-γ signal, showed that this tumor inhibitory effect of IFN-γ is primarily at the level of the tumor cell rather than the host (17). IL-12 also facilitates the differentiation of T_H0 to T_H1 and T_H2 to T_H0 (12), and how this contributes to the antitumor activity of IL-12 needs definition. Another possibility that should be explored is the influence of IL-12 and IFN-γ on a population of $CD4^+$ T cells that plays a critical immunosuppressive role in tumor growth. North and Bursuker (18) have distinguished two phases in the progressive growth of antigenic tumors: (*i*) an initial phase from day 1 to day 7 associated with the induction of $CD8^+$ T cells capable of transferring specific resistance to secondary donors, and (*ii*) a subsequent phase starting on day 7 after tumor injection, in which the protective T-cell response disappears and a $CD4^+$ cell population mediating specific suppression appears. In accordance with this view, we have found that the growth of established Meth A is partially suppressed by treatment with anti-CD4 antibodies given on day 7, whereas tumor growth is not affected by anti-CD4 antibodies given at the time of tumor transplantation. Possibly, IL-12 or IL-12-induced products like IFN-γ exert their antitumor effects by inhibiting or reversing the $CD4^+$ T-cell-dependent immunosuppressive state. In contrast to the role of $CD4^+$ T cells in facilitating tumor growth, antibody depletion tests show that $CD4^+$ T cells are involved in the protective effect of the p53 vaccine and low-dose IL-12. The $CD4^+$ T-cell dependence of this low-dose effect of IL-12 distinguishes it from the $CD4^+$ T-cell-independent antitumor effect of higher doses of IL-12 alone. Clearly, much emphasis should be given to understanding the complex role of $CD4^+$ T cells in tumor growth and tumor immunotherapy.

Like other cytokines, IL-12 is not without toxic effects. Gately *et al.* (15) have reported that IL-12 causes anemia, muscle necrosis, and hepatotoxicity in mice. We have found that multiple injections of 500 ng or more of IL-12 (8 injections over 2 weeks) cause weight loss, piecemeal necrosis of the liver, hepatosplenomegaly, and elevation of a number of serum enzymes, including aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and creatine kinase. These changes in lactate dehydrogenase and creatine kinase persist for up to 4 weeks after IL-12 injections have been stopped and can be seen in mice treated with doses of IL-12 as low as 0.1 ng. Much caution will need to be exercised as the potent antitumor effects of IL-12 are explored in the clinic, either as a single agent or as an adjuvant to peptide/protein vaccines.

We thank Dr. M. Fleisher and Dr. M. P. Sanz-Moncasi for valuable contributions to this study.

1. Boon, T., Cerottini, J.-C., Van den Eynde, B., van der Bruggen, P. & Van Pel, A. (1994) *Annu. Rev. Immunol.* **12**, 337–365.

2. Golumbek, P. T., Lazenby, A. J., Levitsky, H. I., Jaffee, L. M., Karasuyama, H., Baker, M. & Pardoll, D. M. (1991) *Science* **254,** 713–716.
3. Mandelboim, O., Berke, G., Fridkin, M., Feldman, M., Eisenstein, M. & Eisenbach, L. (1994) *Nature (London)* **369,** 67–71.
4. Noguchi, Y., Chen, Y.-T. & Old, L. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3171–3175.
5. Kensil, C. R., Patel, U., Lennick, M. & Marciani, D. (1991) *J. Immunol.* **146,** 431–437.
6. Newman, M. J., Wu, J.-Y., Gardner, B. H., Munroe, K. J., Leombruno, D., Recchia, J., Kensil, C. R. & Coughlin, R. T. (1992) *J. Immunol.* **148,** 2357–2362.
7. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chan, S., Loudon, R., Sherman, F., Perussia, B. & Trinchieri, G. (1989) *J. Exp. Med.* **170,** 827–845.
8. Stern, A. S., Podlaski, F. J., Hulmes, J. D., Pan, Y. E., Quinn, P. M., Wolitzky, A. G., Familletti, P. C., Stremlo, D. L., Truitt, T., Chizzonite, R. & Gately, M. K. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 6808–6812.
9. Brunda, M. J., Luistro, L., Warrier, R. R., Wright, R. B., Hubbard, B. R., Murphy, M., Wolf, S. F. & Gately, M. K. (1993) *J. Exp. Med.* **178,** 1223–1230.
10. Nastala, C. L., Edington, H. D., Mckinney, T. G., Tahara, H., Nalesnik, M. A., Brunda, M. J., Gately, M. K., Wolf, S. F., Schreiber, R. D., Storkus, W. J. & Lotze, M. T. (1994) *J. Immunol.* **153,** 1697–1706.
11. Hsieh, C.-S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. & Murphy, K. M. (1993) *Science* **260,** 547–549.
12. Manetti, R., Parronchi, P., Giudizi, M. G., Piccinni, M.-P., Maggi, E., Trinchieri, G. & Romagnani, S. (1993) *J. Exp. Med.* **177,** 1199–1204.
13. Old, L. J., Boyse, E. A., Clarke, D. A. & Carswell, E. (1962) *Ann. N.Y. Acad. Sci.* **101,** 80–106.
14. Van Pel, A., De Plaen, E. & Boon, T. (1985) *Somat. Cell Genet.* **11,** 467–475.
15. Gately, M. K., Warrier, R. R., Honasoge, S., Carvajal, D. M., Faherty, D. A., Connaughton, S. E., Anderson, T. D., Sarmiento, U., Hubbard, B. R. & Murphy, M. (1994) *Int. Immunol.* **6,** 157–167.
16. Orange, J. S., Wolf, S. F. & Biron, C. A. (1994) *J. Immunol.* **152,** 1253–1264.
17. Dighe, A. S., Richards, E., Old, L. J. & Schreiber, R. D. (1994) *Immunity* **1,** 447–456.
18. North, R. J. & Bursuker, I. (1984) *J. Exp. Med.* **159,** 1295–1311.