

Intratumoral Therapy with IL13-PE38 Results in Effective CTL-Mediated Suppression of IL-13R α 2-Expressing Contralateral Tumors

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Abstract **Purpose:** IL13-PE38, a targeted cytotoxin comprised of interleukin 13 (IL-13) and a mutated form of *Pseudomonas* exotoxin, induces specific killing of tumor cells expressing abundant levels of the IL-13R α 2 chain. We hypothesized that tumor cells killed by the cytotoxin may release antigens and/or apoptotic bodies when cells are dying, which then induce adoptive immunity, and that the PE38 portion of IL13-PE38 may act as a stimulant for the induction of a CTL response. **Experimental Design:** To test this hypothesis, we established D5 melanoma tumors with or without expression of the IL-13R α 2 chain in both flanks of C57BL/6 mice, and then IL13-PE38 was injected in the right flank tumors only. **Results and Conclusions:** When animals with IL-13R α 2-expressing D5 tumor (right) were injected with IL13-PE38, right flank tumors expressing the IL-13R α 2 chain not only showed dramatic regression but contralateral tumors (left flank) also showed tumor regression. Cell depletion experiments in tumor-bearing animals indicated that both CD8⁺ and CD4⁺ T cells contribute to the regression of contralateral tumors through CTL activation in the periphery and cellular infiltration into tumors. In addition, intratumoral treatment into s.c. tumors of mice bearing metastatic lung tumors with IL13-PE38 showed not only the reduction of treated s.c. tumor but also the reduction of lung metastasis. Thus, IL13-PE38 mediates an antitumor effect not only directly but also indirectly by inducing a host CD8⁺ T cell immune response. Accordingly, targeted cytotoxins may be used to treat local disease even if they cannot be administered systemically, and yet may still induce a reasonable systemic antitumor response.

A new form of cancer therapy has evolved over the last two decades by using unique or overexpressed cell surface antigens or receptors on tumor cells as therapeutic targets. It has been shown that neutralizing monoclonal antibodies to some of these antigens or receptors, either alone or coupled to radionuclide, can inhibit the growth of tumor cells by evoking host immune responses (1, 2). For direct tumor cell killing, antibodies or ligands have been chemically conjugated or genetically fused to plant or bacterial toxins, and these forms of therapeutics are termed cytotoxins or immunotoxins (3–7).

Among numerous tumor cell surface-associated molecules, the interleukin 13 receptor (IL-13R) α 2 chain is overexpressed on certain types of human cancer cells including glioblastoma, head and neck cancer, kidney cancer, and Kaposi's sarcoma (8–15). This protein is one of the two subunits of the receptor for IL-13, a Th2 cell-derived pleiotropic immune regulatory cytokine (15). The IL-13R α 2 chain has not been shown to mediate signal transduction through the JAK-STAT pathway; however, it strongly binds IL-13 and induces internalization of this cytokine (15, 16). It has been hypothesized that the extracellular domain of IL-13R α 2 chain might serve as a decoy for the IL-13/IL-13R system (17, 18). Further investigations on the IL-13R α 2 chain have revealed its role in lung epithelial cells (19) and fibroblasts (20) in the context of inflammatory diseases.

To target IL-13R, a recombinant fusion IL-13 cytotoxin termed IL13-PE38QQR, IL13-PE38, or IL13-PE was developed (13, 21, 22). This protein is composed of human IL-13 and a mutated form of *Pseudomonas* exotoxin (PE), which is a 66 kDa molecule secreted by *Pseudomonas aeruginosa* that irreversibly ADP-ribosylates the diphthamide residue of elongation factor 2, using NAD⁺ as a cofactor (3, 4). As a consequence, protein synthesis is inhibited and cell death occurs. IL13-PE38 cytotoxin has potent antitumor activity in IL-13R-expressing tumor cells *in vitro* (13, 21, 22) and *in vivo* (23–25). After the successful completion of several phase I/II clinical trials, currently this molecule is being tested in a multicenter phase III clinical trial ("PRECISE" study) in patients with recurrent glioblastoma.

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Because IL13-PE38 induces direct killing of cancer cells expressing abundant levels of the targeted tumor antigen, it is possible that tumor cells treated with cytotoxin release tumor antigens and/or apoptotic bodies when cells are dying. These tumor antigens and/or apoptotic bodies may then be taken up by the antigen-presenting cells and presented to host T cells to induce tumor-specific CTLs. In addition, as PE38 is a highly immunogenic component derived from a bacterial toxin, whereas IL-13 is a naturally existing cytokine, it is possible that the PE38 portion but not IL-13 portion, may contribute further as a critical immune stimulant for the induction of a CTL response when tumors are treated with IL13-PE38. To test this hypothesis, we established D5 melanoma tumors in syngeneic C57BL/6 mice with or without expression of IL-13R α 2 chain in both flanks, and then injected the tumor on the right flank only with IL13-PE38. Left flank control tumors were treated with excipient. C57BL/6 mice bearing D5 melanoma tumors were also treated with depleting doses of antibodies to CD4 and/or CD8 prior to treatment with intratumor IL13-PE38 to determine the role of these cells in the host tumor response. Splenic CTL activity and IFN- γ secretion were assayed by chromium release and ELISA, respectively, and tumor infiltration of T cells was determined by immunohistochemical analysis of tumor samples.

Materials and Methods

Cell lines and reagents. D5, a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma (26), was a kind gift of Dr. Bernard A. Fox of the Earle A. Chiles Research Institute, Providence Portland Medical Center, Portland, OR. D5 exhibits low to undetectable class I (H-2 D^b and K^b) expression and no class II expression. Anti-CD4 (GK1.5) and anti-CD8 (2.43) antibodies were generated using hybridoma (American Type Culture Collection, Manassas, VA) ascites in BALB/c nude mice and purified on affinity columns. Rabbit anti-asialo-GM1 antibody was purchased from Wako, Osaka, Japan. Recombinant IL13-PE38 was generated following the procedure previously described (22) and diluted in PBS containing 0.2% human serum albumin for all studies.

Stable transfection and selection. pME18S mammalian expression vector encoding human IL-13R α 2 cDNA (8) was used for stable transfection. The flanking sequence of the vector including junction sequences were verified by direct sequencing (ABI Prism 310; Applied Biosystems, Foster City, CA). The resulting construct was expanded in *Escherichia coli* and purified using an endotoxin-free EndoFree Mega kit (Qiagen Inc., Valencia, CA). Plasmid DNA was cotransfected with 0.8 μ g of pPUR selection vector (Clontech Laboratories, Inc., Palo Alto, CA) into semiconfluent D5 cells (8 μ g/60 mm culture dish) using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA; ref. 27). Cells were maintained for 6 weeks in medium containing puromycin (1 μ g/mL), which was replaced every 3 days. Finally, a single IL-13R α 2-overexpressing clone (clone no. 212; designated D5 α 2) was selected for further analysis. The empty vector control (mock) transfected cell lines (designated D5mock) were used for comparison with IL-13R α 2-transfected cells. To reduce the cytotoxic side effects, puromycin was removed at least 7 days before experiments were done.

Radioreceptor binding assay. Recombinant human IL-13 was labeled with ¹²⁵I (Amersham, Arlington Heights, IL), using IODO-GEN reagent (Pierce, Rockford, IL) as previously described (28). For binding experiments, 1 \times 10⁶ cells in 100 μ L binding buffer (RPMI 1640 containing 0.2% human serum albumin and 10 mmol/L HEPES) were incubated with 200 pmol/L of ¹²⁵I-IL-13 with or without increasing concentrations (up to 10 nmol/L) of unlabeled IL-13 at 4°C for 2 hours. Cell-bound and free ¹²⁵I-IL-13 were separated by

centrifugation through a phthalate oil gradient, and radioactivity was determined with a gamma counter (Wallac, Gaithersburg, MD). The number of IL-13Rs and binding affinities were calculated by the LIGAND program (29).

Immunohistochemistry. Immunohistochemistry was done using the Vector ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Tumor or lymph node samples were harvested and fixed with 10% formalin. Paraffin-embedded sections were deparaffinized by xylene treatment and washed with alcohol (100% to 50%) and PBS. Slides were incubated with mouse anti-human IL-13R α 2 (10 μ g/mL; Diaclone, Besancon, France), rat anti-mouse CD4 (1 μ g/mL, MCA1767; Serotec, Oxford, United Kingdom), or rat anti-mouse CD8 (1 μ g/mL, MCA1108G; Serotec) antibodies or isotype control for 18 hours at 4°C. Slides were then developed using 3,3'-diaminobenzidine substrate biotinylated peroxidase reagent (Vector Laboratories) and counterstained with hematoxylin (Sigma-Aldrich, Inc., St. Louis, MO).

Animals and antitumor studies. Four-week-old (~20 g in body weight) female C57BL/6 mice were obtained from the Frederick Cancer Center Animal Facilities (National Cancer Institute, Frederick, MD). The research project was approved by the Center for Biologics Evaluation and Research, Institutional Animal Care and Use Committee. Animal care was in accordance with the guidelines of the NIH Animal Research Advisory Committee. D5 tumor models were established in the mice by s.c. injection of cells (5 \times 10⁵) in 150 μ L of PBS into the flank. Palpable tumors developed within 3 to 4 days. Tumors were measured by Vernier calipers, and tumor size was determined using the following formula: tumor volume (mm³) = (tumor length) \times (tumor width)² \times 0.4. Five to seven mice were used for each group.

For antitumor activity, mice received intratumoral administration (30 μ L/tumor) of IL13-PE38 (dose, 250 μ g/kg) or excipient on days 4, 5, and 6. PBS including 0.2% human serum albumin was used as an excipient control. In some experiments, D5 tumors were developed in both the right and left flanks of mouse and only the right tumor received injections.

For lung metastasis studies, mice received both s.c. (5 \times 10⁵) and i.v. (5 \times 10⁴) injections of D5mock or D5 α 2 tumor cells. S.c. tumors were injected with IL13-PE38 or excipient on days 4, 5, and 6. Lung samples were harvested from mice, fixed with formalin on day 21, and tumor nodules were counted as previously described (30).

Depletion experiments. For CD4⁺ and CD8⁺ depletion, mice were injected i.p. with 0.25 mg of anti-CD4 antibody (GK1.5) and/or anti-CD8 antibody (2.43) at -2, -1, and +7 days relative to the tumor implantation. Depletion of CD4⁺ and/or CD8⁺ T cells was confirmed by fluorescence-activated cell sorting analysis using blood samples from the treated mice. In some experiments, natural killer (NK) cells were depleted with rabbit anti-asialo-GM1 antibody (50 μ g/injection) at days -2, -1, 4, 10, and 16 relative to tumor implantation (31).

ELISA for murine IFN- γ . The concentration of murine IFN- γ in the culture supernatant was determined by ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. The absorbance of the test sample was converted to picograms per milliliter based on a standard curve.

CTL assay. Spleen cells from IL13-PE38-treated mice (4 \times 10⁶ per well) were restimulated with 2 \times 10⁵ mitomycin C-treated D5mock or D5 α 2 tumor cells with IL-2 (20 IU/mL) for 1 week in 24-well plates and then used as effector cells for ⁵¹Cr release. Target cells (3,000/well) labeled with ⁵¹Cr and washed thrice were then incubated with restimulated cells for 4 hours. The mean of triplicate samples was calculated, and the percentage of specific lysis was determined using the following formula: percentage of specific lysis = 100 \times (experimental ⁵¹Cr release - spontaneous ⁵¹Cr release) / (maximum ⁵¹Cr release - spontaneous ⁵¹Cr release). The maximum release refers to counts from targets in 5% SDS.

Statistical analysis. The data were analyzed for statistical significance using Student's *t* test. All statistical tests were two-sided.

Results

Characterization of IL-13R α 2 in D5 α 2 tumor cells. D5 cell clones stably transfected with IL-13R α 2 were characterized for IL-13R α 2 mRNA expression by reverse transcription-PCR analysis (data not shown) and protein expression by radio-receptor binding assays. IL-13 binding sites per cell on clone no. 212, designated D5 α 2, was determined to be 52,700 sites/cell with a K_d value of 19.7 nmol/L (Fig. 1A). To evaluate the persistence of IL-13R α 2 expression *in vivo*, transfected D5 α 2 tumor cells were implanted in C57BL/6 mice and their growth and IL-13R α 2 expression was monitored. Tumors were harvested on days 5, 10, 15, and 20 after implantation and samples were analyzed by immunohistochemical staining to assess the IL-13R α 2 protein expression. As shown in Fig. 1B, IL-13R α 2 expression level decreased as tumors grew. The protein expression declined to 82% of control at day 5 of tumor growth, and further declined to 52% at day 20. As expected, D5mock tumors did not express IL-13R α 2 chain (data not shown).

Effect of IL13-PE38 on D5 α 2 tumor growth and host immune response against D5 α 2 tumor. To assess the effect of IL13-PE38, C57BL/6 mice were implanted with two tumors, D5mock and D5 α 2, in the left and right flanks of mice, respectively. The tumor in the right flank was injected intratumorally with either excipient or IL13-PE38 on days 4 to 6 (Fig. 2A, top). Untreated D5mock or D5 α 2 tumors grew rapidly and tumor volume reached 1,437 to 1,749 mm³ on day 17. On the other hand, the tumor volume of right D5 α 2 tumors receiving IL13-PE38 was 235 to 292 mm³ on day 17 in contrast to excipient-treated control tumors (1,348-1,516 mm³).

When tumors in both flanks expressed the IL-13R α 2 chain, mice treated with excipient showed similar tumor growth reaching 1,526 mm³ comparable to that of tumors not expres-

sing IL-13R α 2 (mock-transfected control; 1,437-1,749 mm³; Fig. 2A, middle). However, when the right tumors were treated with IL13-PE38, the mice showed not only a dramatic reduction of the injected tumor to only 235 mm³, but also the concomitant reduction of contralateral untreated tumors to 664 mm³. The reduction of IL13-PE38-treated tumors (Fig. 2A, middle right) compared with the excipient control was statistically significant ($P < 0.05$). In addition, reduction of the contralateral (left), untreated tumor in mice in which the right tumor was treated (Fig. 2A, Δ , middle right) was also statistically significant ($P < 0.05$) compared with the untreated control (Fig. 2A, Δ , middle left; 1,526 mm³).

Some mice also received excipient or IL13-PE38 in mock control tumors (in the right flank), whereas D5 α 2 tumors (in the left flanks) were left untreated (Fig. 2A, bottom). Although excipient-treated tumors and contralateral D5 α 2 tumors did not show any difference in growth, mock tumors treated with IL13-PE38 showed statistically significant reduction of tumor growth ($P < 0.05$) compared with excipient-treated mock tumors. This may be a direct effect of the IL13-PE injected into the tumor. However, the D5 α 2 tumor treated with IL13-PE38 (top right) was still significantly smaller than the mock tumor treated with IL13-PE38 (bottom right), indicating that the efficacy of IL13-PE38 is increased by targeting through its receptor IL-13R α 2 chain. These results suggest that untreated contralateral D5 tumors showed a regression of tumors only when both tumors expressed IL-13R α 2 chain and right tumors were treated with IL13-PE38, indicating that the IL13-PE38 was not entering the circulation and acting on the uninjected tumor. Rather, it suggests that a specific host immune response was responsible for the effect on the contralateral D5 α 2 tumors.

We then injected D5 α 2 tumor cells *i.v.* into mice at the same time as they received *s.c.* either D5mock or D5 α 2 tumor cells. Mice then received intratumoral IL13-PE38 or excipient in *s.c.* tumors on days 4 to 6, and tumor growth was observed

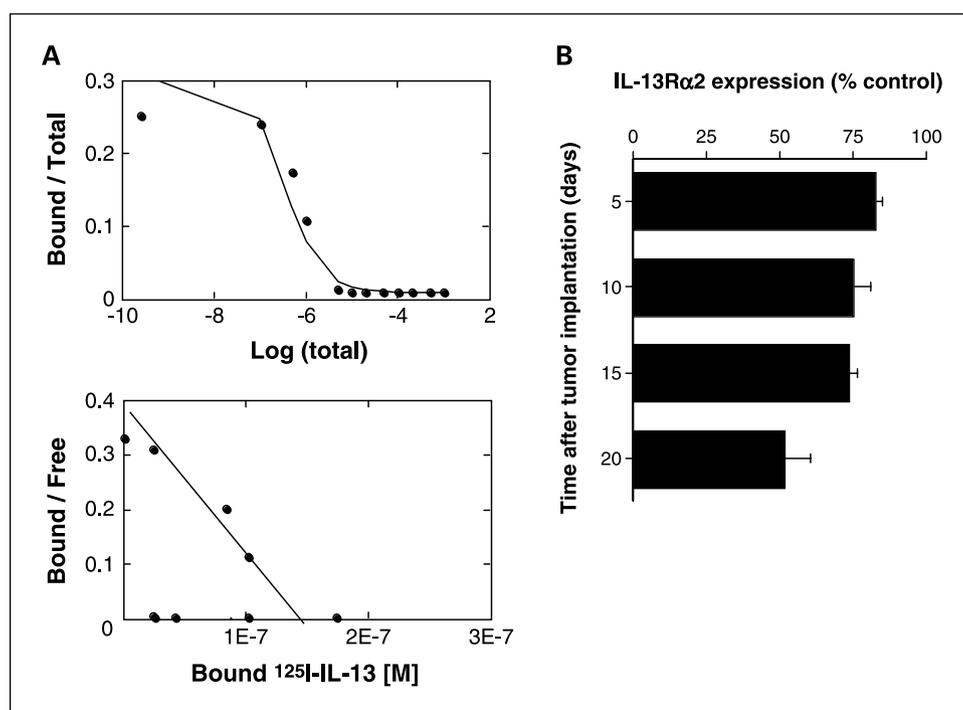
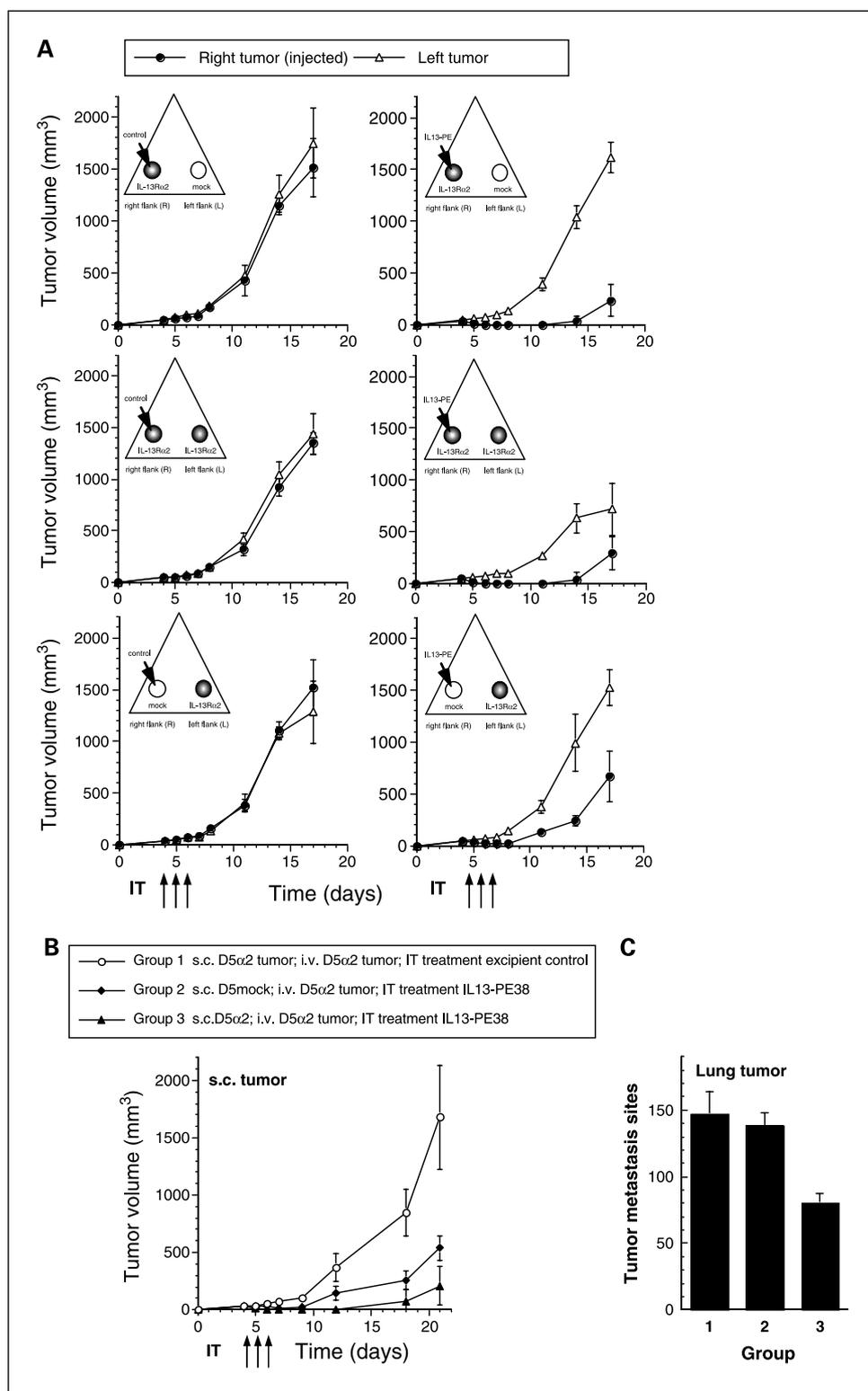


Fig. 1. IL-13 receptor α 2 chain expression in D5 α 2 cells. **A**, D5 α 2 cells (clone no. 212; 1×10^6) were incubated with ¹²⁵I-IL-13 (200 pmol/L) with increasing concentrations of unlabeled IL-13 at 4°C for 2 hours. The displacement curve (top) and Scatchard plot (bottom) were generated from binding data using the LIGAND program. **B**, D5 α 2 tumors implanted *s.c.* in C57BL/6 mice were harvested on days 5, 10, 15, and 20 after tumor implantation. Tumor samples from three animals in each group were subjected to immunohistochemical staining to analyze the expression of IL-13R α 2.

Fig. 2. Effect of IL13-PE38 on tumor growth and host immune response against D5 α 2 tumor. *A*, C57BL/6 mice were implanted with D5mock or D5 α 2 tumors on the left and right flanks and only the right tumor was intratumorally injected with either excipient or IL13-PE38 on days 4 to 6. *B*, D5 α 2 tumor cells were injected i.v. in C57BL/6 mice at the same time as they received s.c. injections with either D5mock or D5 α 2 cells. Mice then received intratumoral IL13-PE38 in s.c. tumors on days 4 to 6, and tumor growth was observed. The mice were sacrificed on day 21 after implantation, and lung samples were harvested to assess the number of tumor nodules (*C*). Each group contained five to six mice, and experiments were repeated four times.



(Fig. 2B). Mice that received IL13-PE38 showed growth reduction of D5 α 2 s.c. tumors. Interestingly, s.c. mock tumors were also reduced in size compared with those in excipient-treated mice, consistent with a direct effect of IL13-PE38 on the injected tumor, although the size of D5 α 2 tumors was even smaller than that of mock tumors. These mice were sacrificed on day 21 after implantation, and lungs were

harvested to assess the number of tumor nodules. As shown in Fig. 2C, the number of D5 α 2 lung tumor nodules was significantly lower in mice receiving s.c. D5 α 2 tumors that were treated with IL13-PE38 (81 ± 7), when compared with mice receiving s.c. D5mock tumors that were treated with IL13-PE38 (138 ± 9) or D5 α 2 tumors without treatment (148 ± 16). As observed in Fig. 1, D5 α 2 cells in lung might

gradually lose IL-13R α 2 expression as the tumor grows, resulting into modest tumor response. These results support the involvement of host immunity towards lung D5 α 2 tumors when the same mice bear s.c. D5 α 2 tumors that were treated with IL13-PE38.

CD8⁺ T cells are necessary for IL13-PE-induced tumor growth suppression of contralateral D5 α 2 tumors with help from CD4⁺ T cells. To confirm the involvement of host immunity and to understand the mechanism of the growth suppression of contralateral left flank D5 α 2 tumors when right flank D5 α 2 tumors were treated with IL13-PE38, *in vivo* depletion experiments were done. CD4⁺ T cells, CD8⁺ T cells, and NK cells were depleted in C57BL/6 mice receiving IL13-PE38 in the right flank D5 α 2 tumors. As shown in Fig. 3A, right flank tumors

that received IL13-PE38 on days 4, 5, and 6 showed profound suppression of tumor growth. Depletion of host cells with anti-CD4, anti-CD8, or anti-asialo-GM1 antibodies did not affect the IL13-PE38-mediated tumor growth suppression. The left flank tumors also showed tumor suppression when right flank tumors were treated with IL13-PE38 (Fig. 3B). When mice were depleted of CD4⁺ T cells, no change in tumor growth suppression was observed compared with nondepleted mice. However, when both CD4⁺ and CD8⁺ T cells were depleted, left flank tumors did not show tumor suppression and tumor volumes were similar to right flank untreated tumors. The D5 α 2 tumor volume on day 22 was significantly larger (2,813 mm³) compared with undepleted mice (1,350 mm³; *P* < 0.05) or mice depleted of CD4⁺ cells only (1,454 mm³; *P* < 0.05;

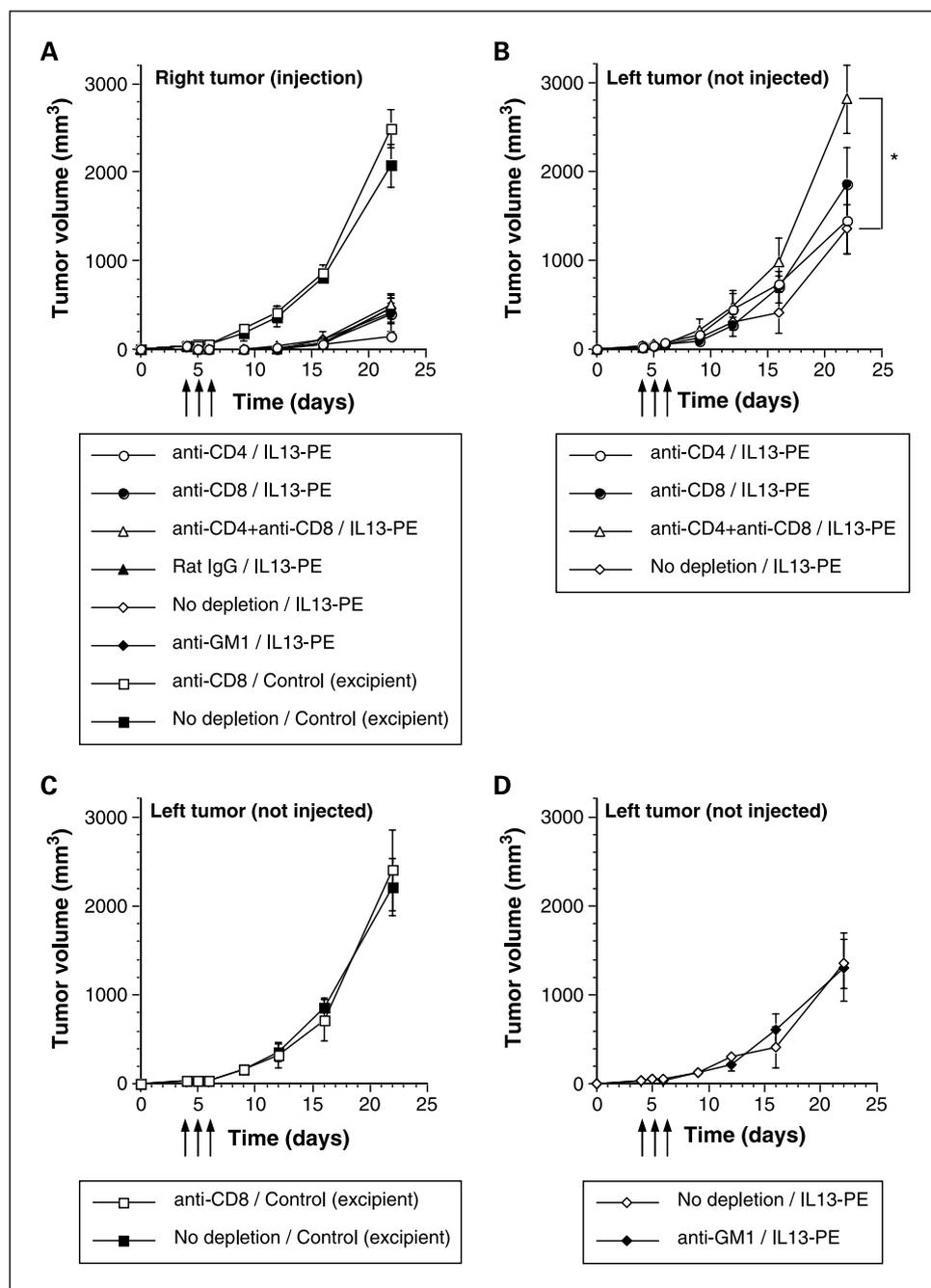


Fig. 3. Role of host immune cells in IL13-PE-induced tumor growth suppression of contralateral D5 α 2 tumors. CD4⁺ and/or CD8⁺ T cells, or NK cells were depleted in C57BL/6 mice receiving IL13-PE38 (days 4-6 after tumor implantation) in right flank D5 α 2 tumors. For CD4⁺ and CD8⁺ depletion, mice were injected i.p. with 0.25 mg of anti-CD4 antibody (GK1.5) and/or anti-CD8 antibody (2.43) at -2, -1, and +7 days relative to the tumor implantation. NK cells were depleted with rabbit anti-asialo-GM1 antibody (50 μ g/injection) at days -2, -1, 4, 10, and 16 relative to tumor implantation. *A*, right flank tumor growth; *B*, left flank tumor growth in CD4 and/or CD8-depleted mice; *C*, left flank tumor growth in CD8-depleted and control mice without IL13-PE38 treatment; and *D*, left flank tumor growth in NK-depleted and control mice. Each group contained five to six mice, and experiments were repeated twice.

Fig. 4. Specific lysis, IFN- γ production, and histology induced by IL13-PE38 treatment. Splenocytes were harvested from mice receiving either intratumoral IL13-PE38 (on days 4-6) or excipient control at day 12 after tumor implantation. For restimulation, D5mock and D5 α 2 cells were treated with mitomycin C (100 μ g/mL) for 1 hour and then washed thrice. **A**, culture supernatants of splenocytes restimulated with D5mock or D5 α 2 cells for 48 hours were assessed for mIFN- γ production by ELISA. Three mice were studied in each group (*columns*). *Columns*, mean of triplicate ELISA wells for each mouse; *bars*, SD. **B**, splenocytes restimulated with D5 α 2 cells for 1 week in culture medium containing IL-2 (20 IU/mL) were used as effector cells. D5mock or D5 α 2 target cells were labeled with 51 Cr for 2 hours, washed thrice, and then plated into 96-well plates with effector cells. Specific lysis was determined after 4 hours of culture. Experiments were done thrice with total of 11 mice and representative data from individual mice of each group are shown. *Bars*, SD; *, $P < 0.05$ control versus IL13-PE at E/T ratio of 50. **C**, histologic analysis of contralateral D5 α 2 tumors from mice receiving IL13-PE38 in right flank D5 tumors. C57BL/6 mice implanted s.c. with tumors on both flanks (D5mock or D5 α 2; day 0) received IL13-PE38 in right flank or excipient control in left flank tumors on days 4, 5, and 6 after implantation. The contralateral left flank tumor samples were collected on days 11, and the immunohistochemistry analysis was done using antibodies specific for CD4 or CD8.

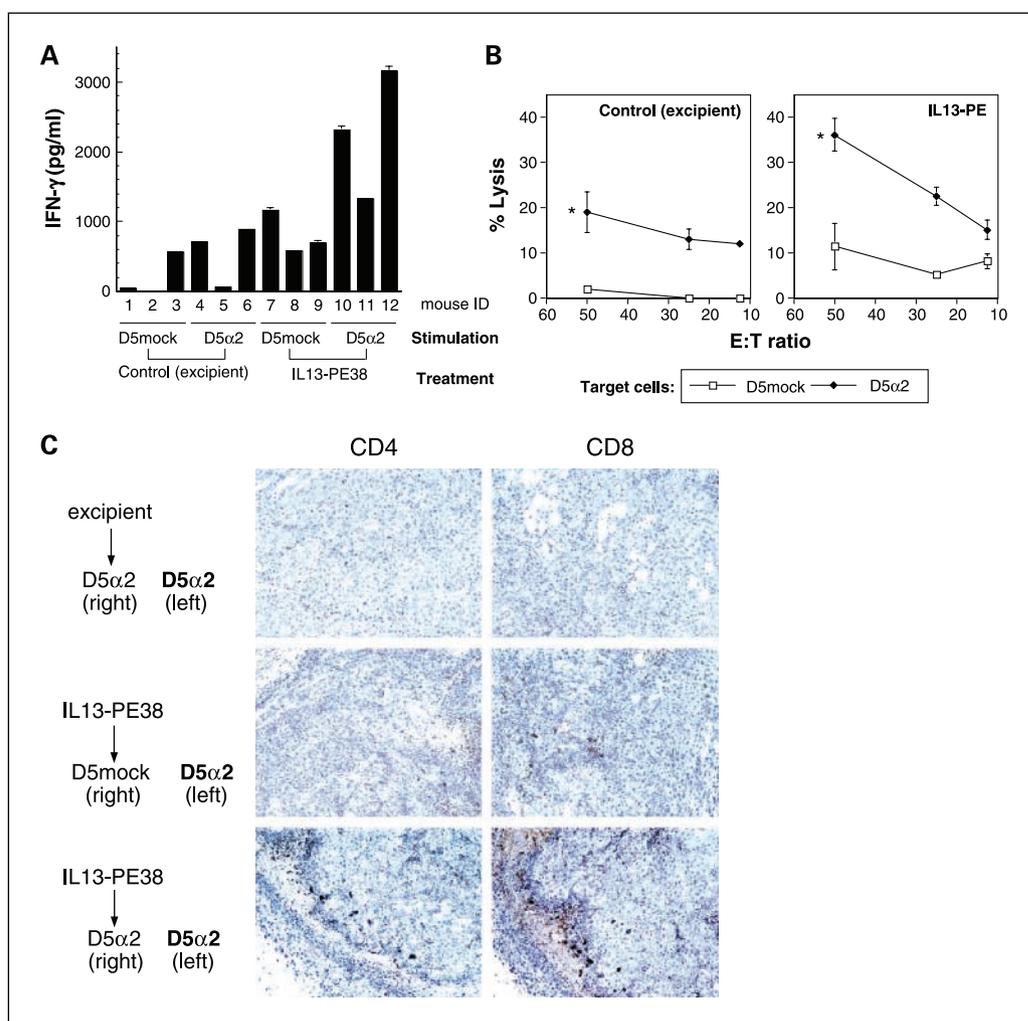


Fig. 3B). However, the left flank tumor volume in mice depleted of CD8 $^{+}$ cells only was only moderately but nevertheless significantly larger (1,857 mm 3 ; $P < 0.05$) than that in undepleted mice (1,350 mm 3 ; $P < 0.05$). When right flank D5 α 2 tumors were treated with excipient only, left D5 α 2 tumor volume was not affected regardless of CD8 $^{+}$ T cell depletion, suggesting IL13-PE38 treatment was necessary to cause T cell-mediated growth suppression of contralateral D5 α 2 tumors (Fig. 3C).

Tumor-bearing mice were also depleted of NK cells and their right flank tumors were treated with IL13-PE38. As shown in Fig. 3D, left flank D5 α 2 tumor volumes in depleted mice were similar to that in undepleted mice.

Taken together, these results suggest that CD8 $^{+}$ T cells, with help from CD4 $^{+}$ T cells, but not NK cells, are critical for the growth suppression of contralateral D5 α 2 tumors when right flank D5 α 2 tumors received intratumoral injections of IL13-PE38.

Intratumoral IL13-PE38 treatment of D5 α 2 tumors induces D5 α 2-specific CTL activity in vivo. We next examined whether the growth suppression of contralateral left flank D5 α 2 tumors when right flank D5 α 2 tumors received intratumoral injections of IL13-PE38 (days 4-6) correlated with the generation of CTL activity *in vivo*. For this study, D5 α 2 tumors were established in both flanks of mice and the

right flank tumors were treated with excipient or IL13-PE38. The spleens from D5 α 2 tumor-bearing mice which did or did not have IL13-PE38 treatment were harvested on day 12 (6 days after the final treatment) and splenocytes were restimulated with D5mock or D5 α 2 tumor cells for 48 hours *in vitro*. These activated splenocytes were used to determine CTL activity by 51 Cr-release assays and culture supernatants of splenocytes were tested for IFN- γ production by ELISA. As shown in Fig. 4A, supernatant from splenocytes obtained from mice treated with IL13-PE38 and restimulated with D5 α 2 cells *in vitro* contained 1,336 to 3,157 pg/mL of mIFN- γ (Fig. 4A, bars 10-12). However, splenocytes from untreated control mice or from treated mice that were restimulated with D5mock cells exhibited comparatively low levels of mIFN- γ (ranging from 55 to 1,170 pg/mL) in culture supernatant (Fig. 4A, bars 1-9).

In CTL assays, splenocytes from the mice treated with IL13-PE38 restimulated with D5 α 2 cells for 1 week mediated specific lysis of D5 α 2 target cells (Fig. 4B). The percentage of lysis was ~36% at an E/T ratio of 50:1. These splenocytes mediated minimum lysis of D5mock target cells. In contrast, splenocytes from D5 α 2 tumor-bearing mice treated with excipient only showed much lower levels of lysis of D5 α 2 target cells (~19% lysis at an E/T ratio of 50:1; $P < 0.05$). These results suggest that treatment of D5 α 2 tumor-bearing mice with IL13-PE38 induces

or amplifies a specific CTL response against the treated tumor expressing target antigen.

Dominant infiltration of CD8⁺ T cells in contralateral D5 α 2 tumors of mice receiving intratumoral IL13-PE38 in right flank D5 α 2 tumors. We also assessed the infiltration of CD4⁺ and CD8⁺ T cells in contralateral D5mock or D5 α 2 tumors of mice receiving IL13-PE38 in their D5 α 2 tumors on the right flank. C57BL/6 mice bearing s.c. D5 tumors in both flanks received IL13-PE38 or excipient only in right flank D5 tumors (D5mock or D5 α 2) on days 4 to 6 after tumor implantation. The left D5 α 2 tumor samples were collected on day 11 (5 days after the end of IL13-PE38 treatment), and an immunohistochemical analysis was done using specific antibodies. As shown in Fig. 4C, high numbers of CD8⁺ cells and modest numbers of CD4⁺ cells were identified in left flank D5 α 2 tumors in mice receiving IL13-PE38 in right flank D5 α 2 tumors (Fig. 4C, bottom). Infiltrating CD8⁺ cells were identified mostly adjacent to or in the necrotic zone of the contralateral tumors. Interestingly, low numbers of CD8⁺ cells were identified in left D5 α 2 tumors in mice receiving IL13-PE38 in right D5mock tumors. In contrast, no detectable levels of CD4⁺ and CD8⁺ cells were observed in left flank D5 α 2 tumors in mice receiving excipient control in right flank D5 α 2 tumors. IL13-PE38 treated right flank D5 α 2 tumors showed CD4⁺ and CD8⁺ cells whereas D5mock tumors are not positive for these cells (data not shown). These results indicate that growth suppression of contralateral D5 α 2 tumors when right flank D5 α 2 tumors received intratumoral injections of IL13-PE38 was associated with infiltration of CD8⁺ T cells.

Mice with D5 α 2 tumors that received IL13-PE38 showed an anamnestic response against D5 α 2 tumors. To evaluate whether C57BL/6 mice bearing s.c. D5 α 2 tumors that were treated with intratumoral IL13-PE38 maintained memory against D5 tumors expressing the IL-13R α 2 chain, either D5mock or D5 α 2 tumors (termed second tumors) were implanted s.c. after complete eradication of the first implanted tumors. Because D5 tumors grew very aggressively in C57BL/6 mice, requiring sacrifice in 3 to 4 weeks, we decided to rechallenge with tumors immediately after the eradication of the first tumor. As shown in Fig. 5A, all the mice remained tumor-free after the intratumoral treatment with IL13-PE38 (days 4-6) until day 20 after the first D5 α 2 tumor implantation. The second tumors, either D5mock or D5 α 2, were implanted on day 8 after the first

tumor implantation (2 days after the final treatment). As shown in Fig. 5B, the growth of the second D5 α 2 tumors expressing IL-13R α 2 chain was significantly slower compared with control D5mock tumors. On day 18 of the second tumor implantation, the mean tumor volume of D5 α 2 tumors (529 mm³) was significantly smaller than D5mock tumors (860 mm³; $P < 0.05$). In contrast, in naïve recipients, D5mock and D5 α 2 tumors grow at comparable rates (Fig. 2A). These results suggest that mice bearing D5 α 2 tumors develop an anamnestic response against tumors bearing the IL-13R α 2 chain after treatment with IL13-PE38.

Discussion

In this work, we show that the host immune response is activated in mice bearing IL-13R α 2-expressing melanoma tumors that were treated with intratumoral IL13-PE38. Intratumoral IL13-PE38 treatment of right flank D5 α 2 tumors induced an immune response against left flank D5 α 2 tumors causing regression of this tumor in mice. Because left flank D5 α 2 tumors in mice that received intratumoral IL13-PE38 in contralateral D5mock tumors did not show suppression of tumor growth, the contralateral D5 α 2 tumor growth suppression was not mediated by the leakage of IL13-PE38 into the circulation. Systemic immunity against D5 α 2 tumors was generated, which was also confirmed by demonstration of inhibition of lung metastasis when only s.c. tumors were treated with IL13-PE38. Although D5mock cells potentially express multiple tumor antigens, interestingly, only IL-13R α 2-positive tumors were inhibited when right flank tumors were treated with IL13-PE38. Similarly, *in vitro* experiments showed IL-13R α 2 exclusivity. The mechanism of these surprising observations is not known. It is possible that IL-13R α 2 acts as a dominant tumor antigen and generates CTL with very high avidity for IL-13R α 2 which do not recognize receptor-negative cells. In addition, rapid growth and proliferation of D5 melanoma tumor cells may inhibit polyclonal tumor response due to the production of inhibitory cytokines such as transforming growth factor- β 1. Alternatively, it is possible that the tumor antigens and apoptotic bodies released from dying cancer cells may initially generate an immune stimulus followed by the generation of regulatory T cells in the tumor. Nevertheless, the PE38 may act as a strong adjuvant for the

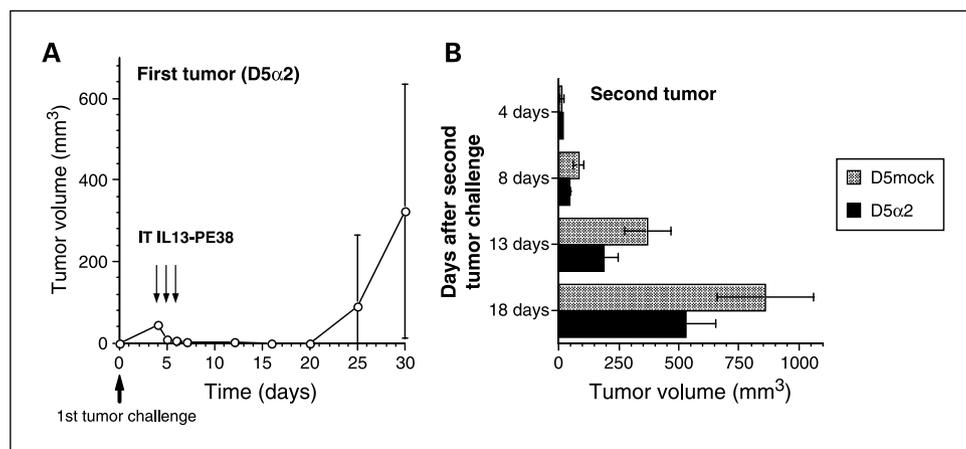


Fig. 5. IL13-PE38 therapy of D5 α 2 tumors induces an anamnestic response in mice. After complete eradication of the first implanted s.c. D5 α 2 tumors by intratumoral IL13-PE38 treatment on days 4 to 6 (A), the second tumors, either D5mock or D5 α 2, were implanted on day 8 after the first tumor implantation (2 days after the final treatment). Each group contained five to seven mice, and experiments were repeated thrice.

generation of a CTL response seen in the contralateral tumors.

It is of interest to note that a slight reduction in the size of mock-transfected tumors was also observed when treated with IL13-PE38. This is likely due to PE38 moiety, which when injected intratumorally, may induce an immune response, even without targeting to a specific receptor. It is probable that similar low levels of tumor inhibition would be seen with free PE38 without the IL-13 moiety, although this has not been tested. In addition, because D5mock cells potentially express multiple tumor antigens, but not IL-13R α 2, perhaps IL13-PE38-mediated lysis of IL-13R α 2-bearing tumors might have also induced an immune response to other shared tumor antigens. Additional studies are necessary to completely explain the underlying mechanism.

The involvement of systemic immunity was confirmed by (a) depletion experiments, (b) induction of tumor-specific CTL response, (c) IFN- γ secretion by splenocytes, and (d) infiltration of CD4⁺ and CD8⁺ T cells in tumors. However, the depletion experiments were not very effective in modulating tumor response caused directly by IL13-PE38 in D5 α 2 right flank tumors possibly because IL13-PE38 itself causes significant tumor lysis after direct injection, which in turn overrode this response. In all four of these studies, CD8⁺ T cells were found to play an important role in contralateral tumor regression. Although CD4⁺ T cells alone did not directly mediate the host immune response against the D5 α 2 tumor, help from CD4⁺ T cells was important for the regression of contralateral D5 α 2 tumors. Asialo-GM1⁺ NK cells were not involved in tumor regression. Splenocytes from treated mice with IL13-PE38 mediated specific lysis of D5 α 2 target cells *in vitro*. These results suggest that either IL-13R α 2 antigen is shed from IL13-PE38-induced dying tumor cells or apoptotic bodies are released from dying cells, or both, and these molecules are taken up by antigen-presenting cells, which in turn activate T cells mediating systemic immunity against D5 α 2 tumors. Based on these conclusions, it is predicted that IL13-PE38 will be effective against systemic metastatic tumors even following treatment of only local tumors.

Cytotoxins are known to be metabolized in the liver and cause dose-limiting reversible hepatotoxicity. However, high local concentration of cytotoxins can be administered without hepatotoxicity, generating systemic therapeutic immunity against the treated tumors. Our results, which generated

systemic immunity by treating local tumors, were further confirmed when animals completely cured of local disease after treatment with IL13-PE38 were challenged with another tumor expressing the IL-13R α 2 chain. These animals showed dramatic slowing of tumor growth compared with control mice, indicating that these animals generated memory to the IL-13R α 2 chain. These results indicate that IL13-PE38 not only causes tumor regression but generates adoptive immune responses preventing tumor recurrence.

It is possible that heterogeneous tumors expressing different levels of receptors will be targeted for CTL-mediated cytotoxicity. In our previous study, we reported that intratumoral administration of plasmid vector expressing IL-13R α 2 gene followed by intratumoral IL13-PE38 administration in nude mice caused rejection of the entire tumor even though not all cells were evenly transduced (27). We showed that an innate immune response was induced which helped in the rejection of tumors. However, in the current study, immunocompetent mice were used and ~100% of tumor cells were transduced prior to the injection. It is possible that as tumors grew, some cells lost IL-13R α 2 gene expression, creating heterogeneity. As we did see significant reduction of tumors in these mice, it is possible that heterogeneous tumors expressing varying densities of IL-13R α 2 will be sensitive to CTL-mediated cytotoxicity. Similarly, we expect that tumor cells expressing natural and perhaps heterogeneous levels of the receptor on tumors such as glioblastoma, head and neck cancer, kidney cancer, and Kaposi's sarcoma will behave similarly to the cell line over-expressing the receptor.

Many immunotherapy approaches, including therapeutic tumor vaccines targeting specific tumor antigens, are being developed based on the understanding of the immunologic and genetic aspects of tumors (32–34). Because IL13-PE38 kills and thereby exposes antigens on IL-13R α 2-expressing tumors to the host immune system, it is possible that an IL-13R α 2 DNA vaccine (35) plus IL13-PE38 treatment may induce a synergistic effect on the growth of IL-13R α 2-positive tumors. These possibilities are currently being investigated in our laboratory.

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