Characterization of a Novel Human Tumor Antigen Interleukin-13 Receptor α2 Chain

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Abstract

The interleukin (IL)-13 receptor α2 (IL-13Rα2) chain is a primary binding and internalization subunit for a Th2-derived immune regulatory cytokine, IL-13. Although extremely high levels of IL-13Rα2 chain are expressed on a variety of human tumor cells and specimens, its precise role in tumor immunology has not been defined. To investigate the role of IL-13Rα2 in tumor immunity, we used D5 melanoma cells stably transfected with the human IL-13Rα2 gene (D5α2) to assess the effect of an IL-13Rα2 DNA vaccine in immunocompetent animals. Prophylactic immunization of mice with the IL-13Rα2 DNA vaccine resulted in protection against D5α2 tumor development. *In vivo* depletion experiments in C57BL/6 and RAG-2 knockout mice indicated that both T and B cells, but not natural killer cells, were required for the tumor protection. In addition, antibody induced by the IL-13Rα2 DNA vaccine showed a modest but significant inhibitory effect on D5α2 cells *in vitro*, suggesting that the antibody is biologically functional. The IL-13Rα2 DNA vaccine also exhibited antitumor activity against established D5α2 tumors in mice. Histologic analysis of regressing tumors identified infiltration of $CD4^+$ and $CD8^+$ T cells and the expression of CXCL9 chemokine in tumors. Taken together, our results identify the human IL-13Rα2 chain as a novel tumor rejection antigen. (Cancer Res 2006; 66(8): 4434-42)

Introduction

It is widely accepted that cancer cells express cell-surface molecules such as specific antigens or cytokine receptors (1–3). These molecules can be used as a target for immunotherapy, cytotoxin/immunotoxin, or gene therapy. Among these various therapeutic approaches against cancer, tumor vaccines are being developed based on the understanding of the immunologic and genetic aspects of tumors (1–3). In contrast to conventional prophylactic vaccines for infectious disease, therapeutic tumor vaccines currently under development are designed to activate the host immune system against existing tumors.

In the past two decades, a major scientific effort has focused on the identification of tumor specific antigens (3). Among numerous tumor cell-surface specific molecules, the interleukin (IL)-13 receptor α2 (IL-13Rα2) chain is reported to be overexpressed on certain types of human cancer cells, including glioblastoma, head and neck cancer, kidney cancer, ovarian cancer, and Kaposi's sarcoma (4–11). This protein is one of the receptor components for IL-13, a Th2 cell-derived pleiotropic immune regulatory cytokine (11). The IL-13Rα2 chain does not mediate signal transduction through the Janus-activated kinase-signal transducers and activators of transcription (STAT) pathway; however, it strongly binds to IL-13 and induces internalization of this cytokine (11, 12). It has been hypothesized that the IL-13Rα2 chain might serve as a decoy for IL-13/IL-13R system (13, 14). Further investigations on the IL-13Rα2 chain have revealed its role in lung epithelial cells (15) and fibroblasts (16) in the context of inflammatory diseases. We have previously reported that overexpression of the IL-13Rα2 chain in pancreatic and breast cancer cells by stable transfection induces reduced tumorigenicity in athymic nude mice, confirming that the IL-13Rα2 chain is involved in the tumor biology (17). In addition, Okano et al. (18) have identified a CTL epitope in the IL-13Rα2 chain by *in vitro* stimulation of dendritic cells with synthetic peptides, implying that this receptor chain might serve as a tumor antigen inducing CTL, which represent a major arm of antitumor immunity. This indirect evidence suggests a role for the IL-13Rα2 chain in cancer biology; however, the significance of IL-13Rα2 expression in the context of cancer immunology is still not clear. Because of the selective expression of IL-13Rα2 in several types of tumors but not in normal tissues, we hypothesized that the IL-13Rα2 chain may serve as a possible novel tumor antigen.

To prove this hypothesis, we evaluated and confirmed the prediction of a prophylactic and therapeutic effect of IL-13Rα2 cDNA vaccination in syngeneic animal models of D5 melanoma stably transfected with the human IL-13Rα2 gene. Depletion and antibody generation experiments in C57BL/6 and RAG-2 knockout mice indicated that not only $CD4^+$ and $CD8^+$ T cells but also antibody against IL-13Rα2 plays an important role in the tumor protection mechanism. Similarly, infiltration of $CD4^+$ and $CD8^+$ T cells and chemokine production are consistent with the ability of effector cells and molecules to play a role in tumor regression mechanisms.

Materials and Methods

Cell lines, DNA vaccine, and reagents. D5, a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma (19), was a kind gift from 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. The human U251 glioblastoma cell line was cultured as previously described (20). The VR1020 mammalian expression vector (a kind gift from Vical, Inc., San Diego, CA) encoding the human IL-13Rα2 chain (termed VRα2) was used as DNA vaccine (21). The sequences of the flanking regions of the junctions were verified by direct sequencing (ABI Prism 310, Applied Biosystems, Foster City, CA), and the resulting construct was expanded in

Note: The views presented in this article do not necessarily reflect those of the Food and Drug Administration.

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Escherichia coli and purified using an endotoxin-free EndoFree Mega kit (Qiagen, Inc., Valencia, CA). 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 a previously described procedure (20) and diluted in PBS containing 0.2% human serum albumin for all studies.

Stable transfection and selection. pME18S mammalian expression vector encoding human IL-13Rα2 cDNA (11) was used for stable transfecton. Plasmid DNA was cotransfected with 0.8 μg of pPUR selection vector (Clontech Laboratories, Inc., Palo Alto, CA) into semiconfluent cells (8 μg/60-mm culture dish) using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the instructions of the manufacturer. Cells were maintained for 6 weeks in medium containing puromycin (1 μg/mL), which was replaced every 3 days. Twenty-five resistant clones isolated with the cloning cylinder (Bel-Art Products, Pequannock, NJ) were characterized for IL-13Rα2 chain expression by reverse transcription-PCR and radioreceptor binding assays. Finally, a single IL-13Rα2-overexpressing clone (clone no. 212; designated D5α2) was selected for further analysis. The vector control (mock)–transfected cell lines (designated D5mock) were used for comparison with IL-13Rα2-transfected cells. To reduce the cytotoxic side effects, puromycin was removed at least 7 days before experiments were done.

Animals and tumor models. 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). Female B6.129S6-Rag2<tm1>N12 (C57BL/6 background) mice were obtained from Taconic Farms (Germantown, NY). 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 (5×10^5) in 150 μL of PBS into the flank. Palpable tumors developed within 3 to 4 days. Tumors were measured by vernier caliper and tumor size was determined using the following formula: tumor volume (mm^3) = (tumor length) × (tumor width)^2 × 0.4. Five to seven mice were used for each group.

Immunization with DNA vaccine. Animals were first anesthetized with ketamine and xylazine, then immunized by i.m. injections of VRα2 or control vector at the indicated days in the muscles of the right (50 μg) and left (50 μg) thighs by using a 50 μL Hamilton syringe (total 100 μg/injection).

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 instructions of the manufacturer. The absorbance of the test sample was compared with the standard curve.

CTL assay. Spleen cells from the immunized mice (4×10^6 per well) were restimulated with 2×10^5 mitomycin C–treated D5mock or D5α2 tumor cells with IL-2 (20 IU/mL) for 1 week in 24-well plates and then used as effector cells for ^{51}Cr release assay. Target cells (3,000 per well) were labeled with ^{51}Cr, washed thrice, and 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 × (experimental ^{51}Cr release − spontaneous ^{51}Cr release) / (maximum ^{51}Cr release − spontaneous ^{51}Cr release). The maximum release refers to counts from targets in 5% SDS.

Depletion experiments. For CD4$^+$ depletion in the induction phase, mice were injected i.p. with 0.25 mg of anti-CD4 antibody (GK1.5) at −2 and −1 days relative to the first immunization followed by weekly injections. For CD4$^+$ and CD8$^+$ depletion in the effector phase, 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 challenge. 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 by pre and post tumor challenge with rabbit anti-asialo GM1 antibody (50 μg/injection) at days −2, −1, 4, 10, and 16 relative to tumor challenge (22).

Protein synthesis inhibition assays. U251 cells (10^4 per well) were cultured in leucine-free medium with or without 1:100 dilution of serum taken from the immunized mice and IL13-PE38 (0.1 ng/mL) for 22 hours at 37°C. Then 1 μCi of [^{3}H]leucine (NEN Research Products, Boston, MA) was added to each well and incubated for an additional 4 hours. Cells were harvested and radioactivity incorporated into cells was measured by a β plate counter (Wallac, Gaithersburg, MD).

Proliferation assays. D5α2 cells (5,000 per well) were cultured for 48 hours with or without various concentrations of serum taken from the immunized mice before [^{3}H]thymidine was added to each well. Cells were cultured for an additional 12 hours. Plates were harvested using a Skatron cell harvester (Skatron, Inc., Sterling, VA) onto filter mats and radioactivity incorporated into cells was measured by a β plate counter.

Immunohistochemistry and immunofluorescence assays. Immunohistochemistry was done using the Vector ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to the instructions of the manufacturer. Tumor samples were harvested and fixed with 10% formalin or snap frozen with optimum cutting temperature compound. Paraffin-embedded sections were deparaffinized by xylene treatment and washed with alcohol (100-50%) and PBS. Slides were incubated with rat anti-mouse CD4 (1 μg/mL; MCA1767, Serotec, Oxford, United Kingdom), rat anti-mouse CD8 (1 μg/mL; MCA1108G, Serotec), goat anti-mouse monokine induced by IFN-γ (MIG/CXCL9) antibody (1 μg/mL; R&D Systems), or rabbit anti-mouse IFN-γ-inducible protein-10 (IP-10/CXCL10; 1 μg/mL; Peprotec, Inc., Rocky Hill, NJ) 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).

For immnofluorescence assays, frozen sections were costained with antibodies for CD4 or CD8. Slides were fixed in acetone at −20°C for 5 minutes and briefly dried. Nonspecific binding was blocked by treatment with 10% serum for 1 hour, followed by incubation with antibody or isotype-matched control immunoglobulin. Sections were subsequently incubated for 1 hour with secondary antibody conjugated with a FITC tag. After three washes with PBS, slides were dried and layered with Vectashield antifluorescence fading mounting medium (Vector Laboratories) and coverslipped. The slides were viewed in an Olympus IX70 fluorescence microscope using an appropriate filter (Olympus Optical Co., Tokyo, Japan). Images were compiled from sets of three consecutive single optical sections using SPOT INSIGHT V 3.2 software (Diagnostic Instruments, Sterling Heights, MI). Immunohistochemical assays were done two to three times independently with similar results.

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

Results

Protection from tumor development by prophylactic IL-13Rα2 DNA vaccination. We first tested the prophylactic effect of a DNA vaccine expressing the IL-13Rα2 gene. The IL-13Rα2 DNA vaccine or control vector was injected 6 and 2 weeks before D5mock or D5α2 tumor challenge in C57BL/6 mice, and the tumor growth was evaluated. As shown in Fig. 1, animals receiving the IL-13Rα2 vaccine and challenged with D5mock cells showed aggressive tumor growth, which was similar to that in mice receiving no immunization or control vector. In contrast, mice receiving the IL-13Rα2 vaccine and challenged with D5α2 tumor cells exhibited no tumor growth until day 10. Although tumors began to grow slowly after day 14, the mean tumor volume in these mice at day 23 (178 mm^3) was significantly smaller than that of D5α2 tumors in control mice (2,218 mm^3; $P < 0.05$). These results suggest that IL-13Rα2 DNA vaccination specifically protects against the growth of D5 tumors expressing the IL-13Rα2 chain but not that of the original D5 tumors without the IL-13Rα2 chain.

IL-13Rα2 DNA vaccination induces CTL against D5α2 cells. To assess whether the D5α2 tumor protection caused by prophylactic IL-13Rα2 vaccination was mediated by CD8$^+$ T cells, IFN-γ production and CTL assays were done. Splenocytes

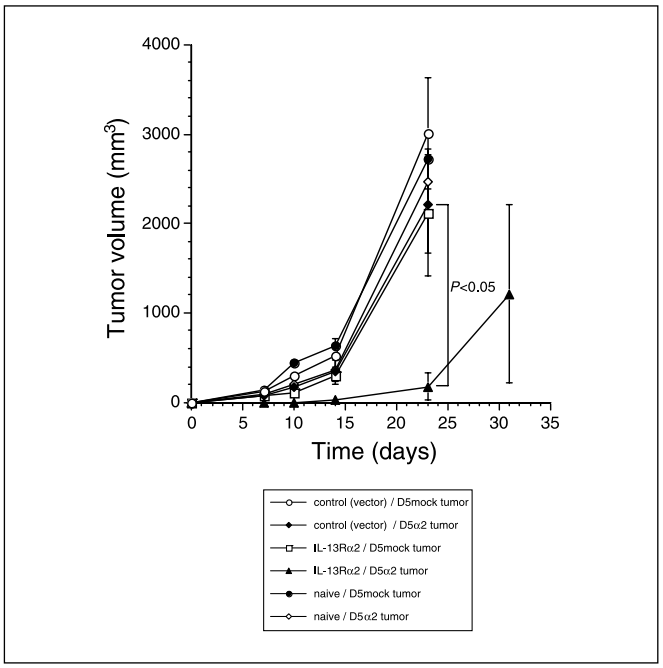

Figure 1. Prophylactic IL-13Rα2 DNA vaccine mediates protection against D5α2 tumors. The VRα2 DNA vaccine or control vector (100 μg) was injected i.m. 6 and 2 weeks before D5mock or D5α2 s.c. tumor challenge (5×10^5 cells per mouse) in C57BL/6 mice, and the tumor growth was evaluated. Experiments were repeated twice; *bars,* SD.

harvested from mice receiving either the IL-13Rα2 DNA vaccine or the control vector at −6 and −2 weeks without tumor challenge were restimulated with mitomycin C–treated D5mock or D5α2 cells and culture medium was tested for cytokine release. When restimulated with D5α2 cells for 48 hours, it was found that culture supernatants of splenocytes from mice immunized with IL-13Rα2 DNA vaccine contained 1,104 to 1,383 pg/mL of murine IFN-γ (Fig. 2*A, columns 10-12*). On the other hand, splenocytes from control mice or mice immunized but restimulated with D5mock cells exhibited low levels (range, 19.8-363 pg/mL) of murine IFN-γ in 48-hour culture supernatant (Fig. 2*A, columns 1-9*).

A CTL assay was done using splenocytes restimulated with D5α2 cells for 1 week in the culture medium containing IL-2. As shown in Fig. 2*B*, control splenocytes barely mediated specific lysis of D5mock target cells (percent lysis ranging from 6% to 8% at an E/T ratio of 50:1). In contrast, splenocytes from mice immunized with IL-13Rα2 DNA vaccine mediated specific lysis of D5α2 target cells. Percent lysis ranged between 18% and 21% at an E/T ratio of 50:1 and percent lysis showed linear decrease when E/T ratio also decreased. These results suggest that immunization with the IL-13Rα2 DNA vaccine generates CTL activity specific for D5α2 cells expressing IL-13Rα2 *in vivo*.

Depletion of both $CD4^+$ and $CD8^+$ T cells is necessary to abrogate tumor protection mediated by IL-13Rα2 DNA vaccination. To clarify the exact mechanism of IL-13Rα2 DNA vaccine–induced protection from D5α2 tumor development, depletion experiments were done. $CD4^+$ and $CD8^+$ T cells and NK cells were depleted in the effector phase in C57BL/6 mice receiving prophylactic IL-13Rα2 DNA vaccination. IL-13Rα2 DNA–vaccinated mice that received either anti-CD4 or anti-CD8 antibody at the time of tumor challenge were protected, indicating that depletion of either one alone did not prevent protection (Fig. 3*A*). It was necessary to deplete both $CD4^+$ and $CD8^+$ T cells to abrogate tumor protection mediated by IL-13Rα2 DNA vaccination (Fig. 3*A*). Thus, either CD4 or CD8 T cells were sufficient to protect. Although the mean tumor volume of D5mock tumors in mice without depletion (1,755 mm^3) was larger than the mean volume of D5α2 tumors in mice treated with both anti-CD4 and anti-CD8 antibodies (1,043 mm^3) at day 19, the difference between these two groups failed to show statistical significance ($P = 0.086$). We also depleted NK cells in the effector phase, but NK depletion did not reduce the protective effect of IL-13Rα2 DNA vaccination on D5α2 tumor growth (Fig. 3*B*). These results suggest that both $CD4^+$ and $CD8^+$ T cells, but not NK cells, are critical in the effector phase for the IL-13Rα2 DNA vaccination-induced protection against D5α2 tumor growth.

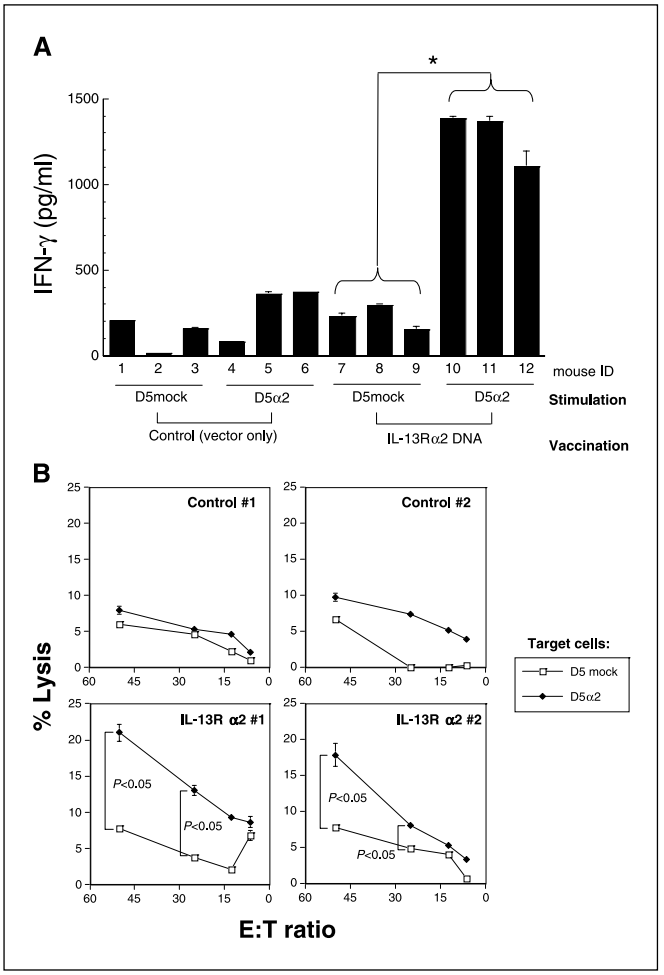

Figure 2. Specific lysis and IFN-γ production mediated by IL-13Rα2 vaccine. Splenocytes harvested from mice receiving either the IL-13Rα2 vaccine or the control vector (100 μg) at −6 and −2 weeks without tumor challenge were prepared for measurement of IFN-γ and CTL assays. For restimulations, D5mock and D5α2 cells were treated with mitomycin C (100 μg/mL) for 1 hour and then washed thrice. *A,* culture supernatants of splenocytes restimulated with D5mock or D5α2 cells for 48 hours were assessed for murine IFN-γ production by ELISA. *, $P < 0.05$, comparing mice vaccinated with IL-13Rα2 vaccine and splenocytes stimulated with D5α2 cells to mice vaccinated with IL-13Rα2 and splenocytes stimulated with D5 mock cells. *B,* splenocytes restimulated with D5α2 cells for 1 week in culture medium containing IL-2 (20 IU/mL) were used as effector cells. D5mock or D5α2 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 calculated as described in Materials and Methods after 4 hours of culture. Experiments were done thrice with a total of 16 mice. Representative data from two individual mice of each group. *Bars,* SD. *P* values compare specific lysis at two E/T ratios using D5α2 and mock tumor as target.

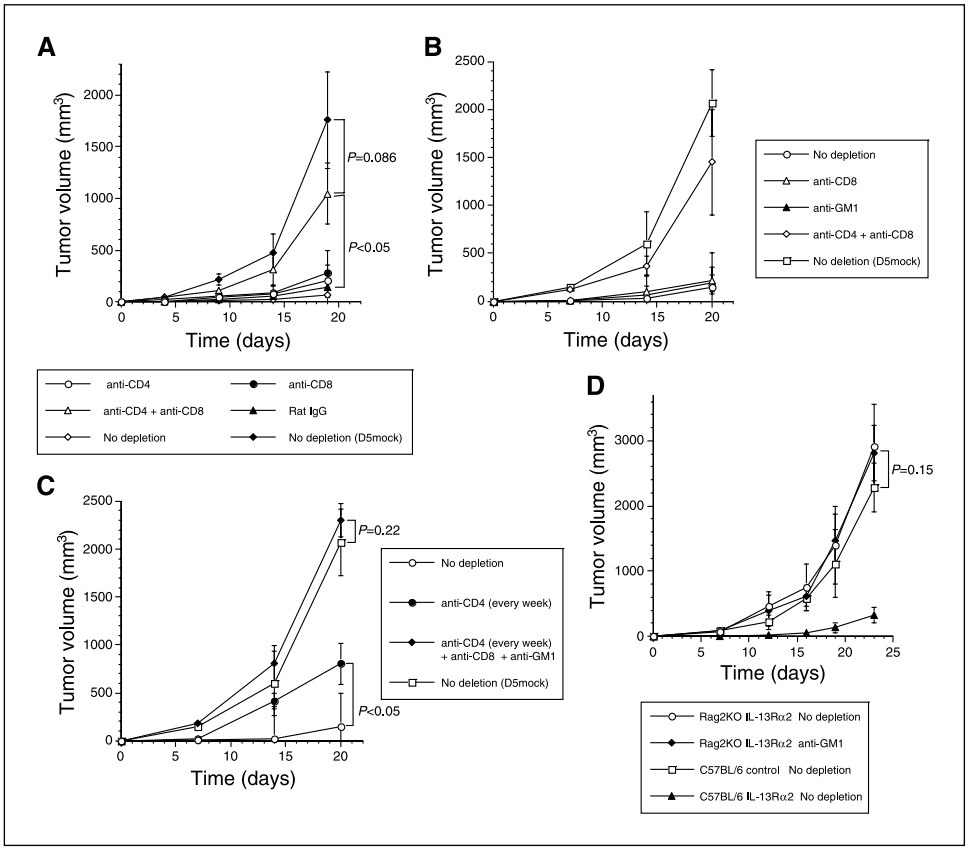

Figure 3. Identification of cells responsible for tumor protection by the IL-13Rα2 DNA vaccine. I.m. immunization was done by injections of the VRα2 DNA vaccine or control vector (100 μg) 6 and 2 weeks before D5mock or D5α2 s.c. tumor challenge. *A,* for the depletion only in the effector phase, C57BL/6 mice received i.p. treatment with rat immunoglobulin G (*IgG*) or anti-CD4 and/or anti-CD8 antibodies (0.25 mg) on days −1, −2, and 7 before and after s.c. D5α2 or D5mock tumor challenge. *B,* C57BL/6 mice received anti-CD4 and/or anti-CD8 antibodies (0.25 mg) on days −1, −2, and 7 days, or anti-asialo GM1 antibody (50 μg) on days −2, −1, 4, 10, and 16, relative to tumor challenge with D5mock or D5α2 cells. *C,* for depletion during the induction and effector phase, C57BL/6 mice received anti-CD4 antibody (0.25 mg) on days −2 and −1 before the first immunization, followed by anti-CD4 antibody injections every week. Depletion with anti-CD8 antibody was done by injections on days −1, −2, and 7 relative to challenge and depletion with anti-asialo GM1 antibody was done by injections on days −2, −1, 4, 10, and 16 relative to tumor challenge. *D,* RAG-2 knockout or C57BL/6 mice received anti-asialo GM1 antibody on days −2, −1, 4, 10, and 16 relative to tumor challenge with D5α2 cells. All the experiments were repeated twice with similar results; *bars,* SD.

$CD4^+$ T-cell help for B cells or other effector cells is also involved in the protection induced by IL-13Rα2 DNA vaccination. Because the depletion of $CD4^+$ and $CD8^+$ T cells was not enough to remove the D5α2 tumor protection mediated by IL-13Rα2 DNA vaccination and NK cells also did not seem to be involved in the protection mechanism, we hypothesized that antibody generated in C57BL/6 mice as a result of IL-13Rα2 DNA vaccination might have been involved in the tumor protection mechanisms against D5α2 tumor. Because CD40L-expressing $CD4^+$ T cells activated by B cells are required for the antibody generation, we did *in vivo* CD4 depletion experiments (23). Mice were treated with anti-CD4 antibody every week during the entire immune-induction phase as well as post tumor challenge (effector phase). As shown in Fig. 3*C*, the depletion of $CD4^+$ T cells partially reversed the protective effect of the IL-13Rα2 DNA vaccine. The mean volume of D5α2 tumors in mice receiving anti-CD4 antibody (806 mm^3) was significantly larger than in the mice with no depletion (152 mm^3) at day 20 ($P < 0.05$). Importantly, when mice received anti-CD4 antibody during the entire experimental period in addition to anti-CD8 and anti-GM1 antibodies during the effector phase, D5α2 tumors exhibited linear growth although they received the IL-13Rα2 DNA vaccine. The mean tumor volume of this animal group was 2,301 mm^3 at day 20, which was similar to the mean volume of D5mock tumor (2,070 mm^3) at day 20 ($P = 0.22$). These results suggest that $CD4^+$ T-cell help for B cells or $CD8^+$ T cells is also required in the protection against D5α2 tumor development induced by IL-13Rα2 DNA vaccine.

To further confirm these results, RAG-2 knockout mice with or without NK-cell depletion were immunized with either the IL-13Rα2 DNA vaccine or the control vector. It has been shown that RAG-2 knockout mice are deficient in both T and B cells (24). As shown in Fig. 3*D*, RAG-2 knockout mice receiving the IL-13Rα2 DNA vaccine failed to exhibit the protection against the D5α2 tumor. Anti-GM1 antibody showed no effect on the tumor growth. The mean tumor volume of D5α2 tumors in RAG-2 knockout mice (2,914 mm^3) was even larger than the mean D5α2 tumor volume in C57BL/6 mice without immunization with the IL-13Rα2 DNA vaccine (2,286 mm^3) at day 23, suggesting the existence of T-cell–mediated natural immunosurveillance in immunologically intact mice. However, the mean sizes of tumors in these groups were not statistically different from each other ($P = 0.15$). Given that T-cell depletion alone seemed to only partially abrogate protection, these results suggest that antibody generated by the IL-13Rα2 DNA vaccine is also involved in the tumor protective mechanism. Thus, both T and B cells are required to achieve protection from D5α2 tumor development.

Antibody generated by IL-13Rα2 DNA vaccination modestly inhibits proliferation of D5α2 cells. The results from both CD4 depletion experiment in C57BL/6 mice and vaccination of RAG-2 knockout mice compared with T-cell–depleted mice indicated that antibody against IL-13Rα2 might play a role in the D5α2 tumor protection induced by IL-13Rα2 DNA vaccination. Blood serum samples were periodically collected from C57BL/6 mice immunized with the IL-13Rα2 DNA vaccine and treated with anti-CD4 antibody during the entire experimental period and from control animals, which were both challenged with D5α2 tumor. To measure the antibody levels against IL-13Rα2, neutralization of cytotoxicity by antibody was assessed in an IL-13R-directed cytotoxin assay. The IL-13R-directed cytotoxin IL13-PE38 is a recombinant protein composed of human IL-13 and *Pseudomonas* exotoxin (9), which

mediates a cytotoxic effect on tumor cells expressing the IL-13Rα2 chain (20, 25). For this purpose, we used a human U251 glioblastoma cell line that expresses abundant levels of IL-13Rα2 chain (20, 25). We tested whether the addition of serum from IL-13Rα2 DNA–vaccinated mice blocked the cytotoxic effect of IL13-PE38 on U251 cells. As shown in Fig. 4*A*, protein synthesis of U251 cells was highly inhibited by IL13-PE38 (0.1 ng/mL) when cells were cocultured with serum from naïve mice (*samples 1* and *2*) or from mice immunized with the IL-13Rα2 DNA vaccine without CD4 depletion but before tumor challenge (*samples 3-5*; range, 12-17% of control). In contrast, the protein synthesis inhibition was clearly blocked by the serum obtained 1 or 2 weeks after D5α2 tumor challenge in immunized mice without CD4 depletion (*sample 6*, 60% of control at 1 week; *sample 7*, 69% of control at 2 weeks). Serum from unimmunized animals challenged with D5α2 tumor minimally blocked the protein synthesis inhibition. On the other hand, the serum taken from animals that received anti-CD4 antibody did not alter the protein synthesis inhibition caused by IL13-PE38 (range, 15-22% of control; Fig. 4*A*). These results suggest that the mice protected from D5α2 tumor development generated antibody against IL-13Rα2, and that IL-13Rα2 DNA vaccination is not sufficient for the generation of detectable levels of antibody against IL-13Rα2 but can be boosted by tumor challenge to induce antibody production.

To assess the effect of antibody to IL-13Rα2 generated in the mice protected from D5α2 tumor development, the sera from C57BL/6 mice at preimmunization (Fig. 4*A*; *No depletion, sample 1*) or 2 weeks after receiving the IL-13Rα2 DNA vaccine, followed by D5α2 tumor challenge (Fig. 4*A*; *No depletion, sample 7*), were incubated with D5α2 cells for 48 hours and the cell proliferation was evaluated. At 1:100 dilution, serum taken from mice receiving IL-13Rα2 DNA vaccination followed by D5α2 tumor challenge showed a modest but significant inhibition of D5α2 cell proliferation (Fig. 4*B*). Although serum taken before vaccination also showed inhibition of D5α2 cell proliferation (82% compared with control), proliferation of cells incubated with serum from mice receiving IL-13Rα2 DNA vaccination followed by D5α2 tumor challenge showed significantly higher suppression (67% compared with control) at 1:100 serum dilution ($P < 0.05$). Because of the limited availability of serum and nonspecific serum toxicity, serum concentrations >1:100 were not tested. Taken together, our results indicate that antibody against IL-13Rα2 has a modest inhibitory effect on IL-13Rα2-expressing D5α2 cells.

Effect of the IL-13Rα2 DNA vaccine on established D5α2 tumors *in vivo*. Having identified the protection mechanisms of the IL-13Rα2 DNA vaccine against the D5α2 tumor, we were interested in using this vaccine in animals bearing established D5α2 tumor. S.c. injection of D5α2 cells in C57BL/6 mice resulted in palpable tumors in 3 to 4 days (tumor volume range, 34-40 mm^3). These mice received i.m. injections of IL-13Rα2 DNA vaccine (100 μg/injection) at days 4, 9, 14, and 19 post tumor implantation. As shown in Fig. 5, treatment of mice with the IL-13Rα2 DNA vaccine delayed the D5α2 tumor growth after day 9 following tumor implantation. The tumor growth was suppressed during the treatment with the IL-13Rα2 DNA vaccine; however, tumors started growing again after day 19, when the vaccination was discontinued. Nevertheless, the mean tumor volume of D5α2 tumors in mice receiving IL-13Rα2 DNA vaccine (681 mm^3) was significantly smaller than that of D5α2 tumors in mice receiving vector control (2,521 mm^3) at day 24 ($P < 0.05$). These results suggest that the IL-13Rα2 DNA vaccine is effective on D5α2 tumor not only in the prophylactic setting but also in the established tumor setting.

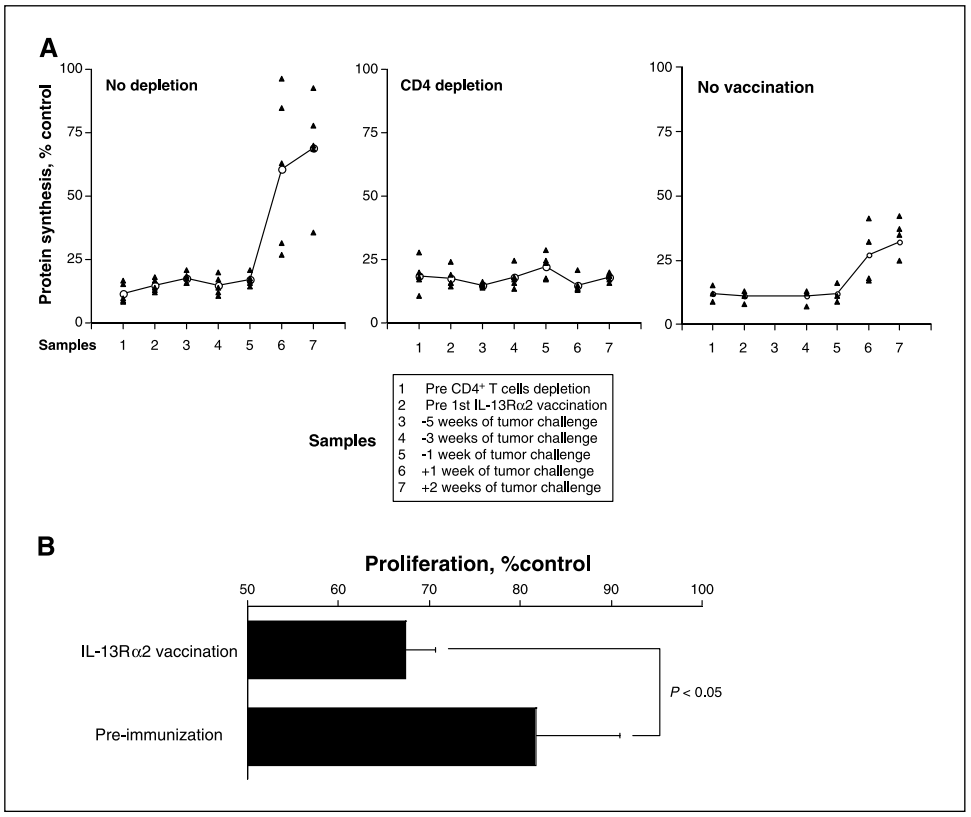

Figure 4. Proliferation of D5α2 cells is inhibited by antibody generated against IL-13Rα2. *A,* to measure the antibody levels in mice, blood serum samples were periodically collected from C57BL/6 mice receiving anti-CD4 antibody during the entire experimental period, no depletion, or no vaccination with D5α2 tumor challenge (treatment schedule as described in the legend to Fig. 3*B*). The U251 glioblastoma cells expressing high levels of human IL-13Rα2 chain were incubated with IL13-PE38 (0.1 ng/mL) with serum (1:100 diluted) for 22 hours to assess the blocking effect of serum on IL13-PE38-mediated protein synthesis inhibition in U251 cells. ○, mean of the value of five individual samples (▲). *B,* proliferation of D5α2 cells incubated with 1:100 diluted serum samples (preimmunization or +2 weeks after tumor challenge from mice without CD4 cell depletion) for 48 hours, followed by additional 12-hour incubation with [^{3}H]thymidine. Experiments were repeated twice; *bars,* SD.

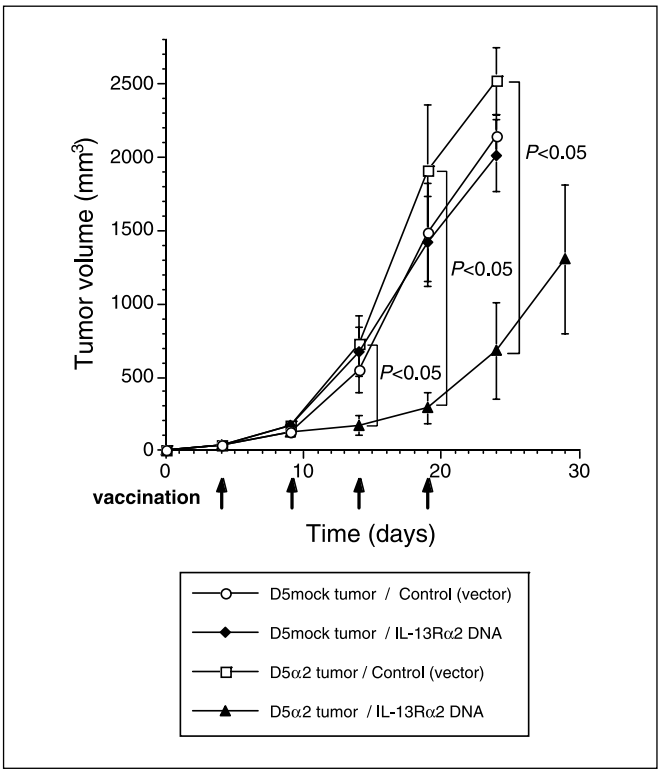

Figure 5. Effect of the IL-13Rα2 DNA vaccine on the growth of established D5α2 tumors. C57BL/6 mice received s.c. injection of 5×10^5 D5mock or D5α2 cells (day 0) and then were treated with the i.m. VRα2 DNA vaccine or control vector (100 μg) on days 4, 9, 14, and 19. Experiments were repeated twice; *bars,* SD.

IL-13Rα2 vaccination induces CTL activity against established D5α2 tumor cells. To assess whether the antitumor effect of the IL-13Rα2 DNA vaccine was associated with induction of CTL against D5α2 tumor, IFN-γ production and lytic activity were examined. For IFN-γ measurement, splenocytes from D5α2 tumor–bearing mice with or without the IL-13Rα2 DNA vaccination were harvested on day 23 (4 days after the final treatment) and restimulated with D5mock or D5α2 tumor cells for 48 hours. The culture supernatants of splenocytes from mice treated with the IL-13Rα2 DNA vaccine restimulated with D5α2 cells contained 1,281 to 1,541 pg/mL of murine IFN-γ (Fig. 6*A, columns 10-12*). In contrast, splenocytes from control mice or cells from treated mice that were restimulated with D5mock cells exhibited comparatively low levels (ranging, 158-756 pg/mL) of murine IFN-γ in culture supernatant (Fig. 6*A, columns 1-9*).

Furthermore, the CTL assay using splenocytes restimulated with D5α2 cells for 1 week showed that splenocytes from the mice treated with IL-13Rα2 DNA vaccine mediated specific lysis of D5α2 target cells (Fig. 6*B*). The percent lysis was 38% at an E/T ratio of 50:1, and decreased with decreasing E/T ratio. These splenocytes did not mediate specific lysis of D5mock target cells. In contrast, splenocytes from D5α2-tumor bearing mice immunized with the control vector showed much lower levels of lysis of D5α2 target cells (19% lysis at an E/T ratio of 50:1). At 20% lysis, there was a 2-fold difference in lytic units between the IL-13Rα2 DNA vaccine and the control (13.3 versus 6.7 LU_{20}/million cells). These results suggest that the treatment of D5α2 tumor–bearing mice with the IL-13Rα2 DNA vaccine induced or amplified a specific CTL response.

Infiltration of $CD4^+$ and $CD8^+$ T cells in tumors of mice receiving the IL-13Rα2 DNA vaccine. Finally, we assessed the infiltration of $CD4^+$ and $CD8^+$ T cells, in addition to IFN-γ–related chemokine (CXCL9 and CXCL10) expression, in established D5α2 tumors of mice receiving the IL-13Rα2 DNA vaccine. C57BL/6 mice bearing D5α2 tumor (day 0) were treated with the IL-13Rα2 DNA vaccine or control vector on days 4, 9, 14, and 19 after tumor implantation. The tumor samples were collected on days 7, 10, 13, 16, and 20 (during or after the completion of the treatment schedule; and three mice from each time period) and an immunohistochemistry analysis was done using specific antibodies. Three sections from each tumor sample were evaluated. CD4-positive cells were identified in tumors of vaccine-treated mice collected on days 7 to 16 with identical number of positive cells, and CD8-positive cells were mainly identified in tumor samples of vaccine-treated mice collected on days 13 and 16. The numbers of CD8-positive cells in samples from day 13 and day 16 are similar. Tumor samples collected from control (vector-injected) mice did not show any positive staining by either anti-CD4 or anti-CD8 antibodies. Both CD4-positive and CD8-positive cells were observed at the highest density in tumor samples collected on day 13 (Fig. 6*C*). The number of CD4-positive cells (per 200× field view and results were average of three view fields) was 0 in control tumor and 45 in IL-13Rα2 DNA–vaccinated mice. On the other hand, the number of CD8-positive cells (per 200× field view) was 6 in control tumor and 126 in IL-13Rα2 DNA–vaccinated mice. The results from immunohistochemistry were further confirmed by immunofluorescent microscopic analysis using frozen samples (data not shown).

Finally, tumor samples were also stained with anti-MIG/CXCL9 or anti-IP-10/CXCL10 antibodies (Fig. 6*D*). CXCL9 and CXCL10 chemokines were selected because they have been shown to be involved in the CTL-induced tumor regression in mice (26–28). Tumor samples of IL-13Rα2 DNA vaccine–treated mice collected on days 13 and 16 were positive for CXCL9 whereas control tumor samples were negative for this chemokine. In contrast, CXCL10 was strongly positive in tumor samples of both control and vaccinated mice collected on days 7 to 16 but higher in vaccinated mice. These results suggest that therapeutic IL-13Rα2 DNA vaccine–induced regression of D5α2 tumor involves infiltration of $CD4^+$ and $CD8^+$ T cells and the production of certain chemokines in tumors.

Discussion

In this study, we have identified and characterized the role of the human IL-13Rα2 chain as a novel tumor antigen. This conclusion is based on (*a*) IL-13Rα2 DNA vaccine–induced protection of animals against challenge with D5α2 tumor expressing human IL-13Rα2 chain *in vivo* and (*b*) regression of established IL-13Rα2-positive D5 tumors by IL-13Rα2 DNA vaccination. Tumor protection mechanisms involved both cellular and humoral immunity, including $CD4^+$ and $CD8^+$ effector T cells, and specific antibody generated against IL-13Rα2.

This is the first report which conclusively shows that the IL-13Rα2 chain, which is uniquely overexpressed on a limited number of types of human tumor cells, is a novel tumor rejection antigen. Splenocytes collected from nonimmunized control mice produced minimal levels of IFN-γ when they were restimulated with D5α2 cells. These splenocytes also mediated low levels of lysis of D5α2 target cells as determined by CTL assays. However, D5α2 cell–restimulated splenocytes collected from mice receiving the

IL-13Rα2 DNA vaccine produced much more substantial levels of IFN-γ in the culture supernatant and they were capable of mediating specific lysis of D5α2 target cells. The involvement of $CD4^+$ and $CD8^+$ T cells in the regressing tumors as determined by T-cell depletion studies was further confirmed by immunohistochemistry. Based on these studies, we conclude that D5α2 tumor protection mediated by the IL-13Rα2 DNA vaccine is human IL-13Rα2 specific.

It is of interest to note that not only $CD4^+$ and $CD8^+$ T cells but also antibody generated against human IL-13Rα2 showed modest direct suppression of D5α2 cell growth. Antibody against IL-13Rα2 was generated in C57BL/6 mice only after a couple of IL-13Rα2 DNA vaccine injections at −6 and −2 weeks before D5α2 tumor challenge but required tumor challenge as a boost. These observations are consistent with previous reports indicating that specific antigen-loaded tumor challenge acts as a booster to enhance levels of specific antibody in the host (3). Although antibodies against tumor antigens including HER2/neu (29) and CD20 (30) are able to mediate direct killing of target cells, it is not known how antibody to IL-13Rα2 induces modest but significant growth inhibition of IL-13Rα2-expressing D5α2 cells. For example, IL-13 has been proposed to play an autocrine role in Hodgkin's lymphoma and blockade of signaling could possibly affect tumor growth (31–34). Further investigations are necessary to unravel the mechanism of action.

It has been unclear why the IL-13Rα2 chain is overexpressed specifically in certain types of solid human cancer cells and how the IL-13Rα2 chain acts as a possible tumor antigen (11). Previously, we have used IL-13Rα2 gene transfer in combination with IL-13 cytotoxin (IL13-PE38) therapy to maximize the effect of IL-13 cytotoxin in less-sensitive tumors (21, 35). However, thus far, no direct evidence has been available about the possible use of this cytokine receptor chain as tumor antigen in immunotherapy. Recently, it has been shown that IL-13 and STAT6 pathways play an important role in tumor immunity (11, 36–39). In addition, it has been shown that transforming growth factor β produced by $CD11b^+$ $Gr\text{-}1^+$ cells, induced by IL-13 and CD1d-restricted NKT cells, is necessary for down-regulation of CTL-mediated tumor immunosurveillance (36, 39). Taken together, these studies show that IL-13 acts as a key player in negatively regulating tumor immunosurveillance, and now we see that its alternate receptor, the IL-13Rα2 chain, can also serve as a tumor antigen.

It is noteworthy that the IFN-γ -related chemokines CXCL9 and CXCL10 were expressed in tumors derived from mice receiving the IL-13Rα2 DNA vaccine. CXCL9 and CXCL10 chemokines were selected because they have been shown to be involved in the

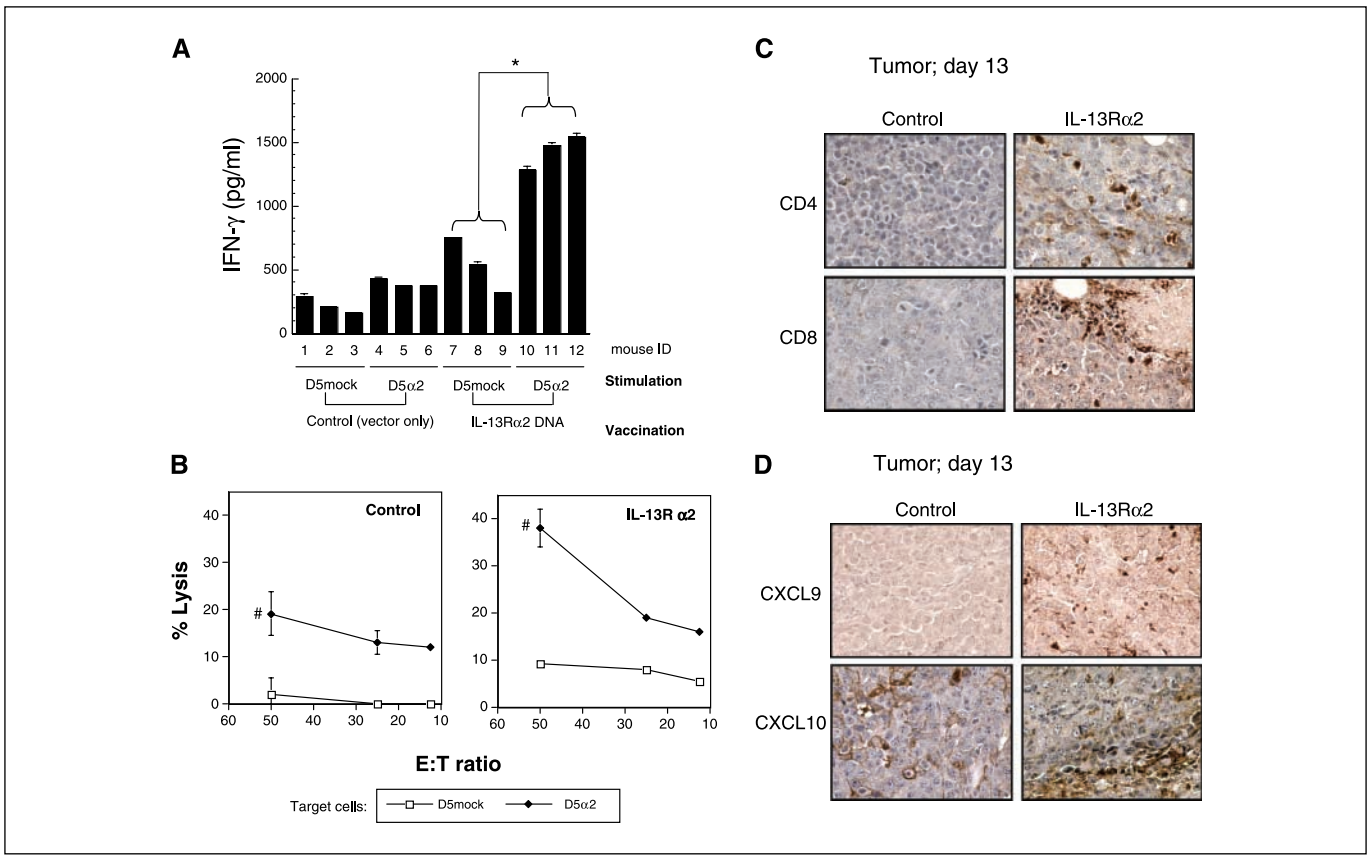

Figure 6. IFN-γ production, CTL lysis, and histopathology in mice bearing established D5α2 tumors mediated by IL-13Rα2 DNA vaccine. C57BL/6 mice received s.c. injection of 5×10^5 D5mock or D5α2 cells (day 0) and then were treated with the i.m. VRα2 DNA vaccine or control vector (100 μg) on days 4, 9, 14, and 19. Splenocytes harvested on day 23 of tumor implantation were prepared for measurement of murine IFN-γ production (*A*) and CTL-mediated specific lysis (*B*) as described in the legend to Fig. 2. Representative data from experiments done four times with a total of 20 mice. *Bars,* SD. *, $P < 0.05$, comparing mice vaccinated with IL-13Rα2 vaccine and splenocytes stimulated with D5α2 cells to mice vaccinated with IL-13Rα2 and splenocytes stimulated with D5 mock cells; #, $P < 0.05$, control versus IL-13Rα2 at an E/T ratio of 50:1. The tumor samples were collected on days 7, 10, 13, 16, and 20 (during or after the completion of treatment schedule) and the immunohistochemistry and immunofluorescence microscopic analyses were done using antibodies specific for CD4 and CD8 (*C*) or CXCL9 and CXCL10 (*D*). Representative data on day 13 tumor samples.

CTL-induced tumor regression in mice (26–28). It has been shown that the CXCL9 functions as a potent chemoattractant for tumor-infiltrating lymphocytes (26). In addition, the CXCL10 has been shown to display antitumor properties based on the attraction of monocytes and T lymphocytes (40). Because both CXCL9 and CXCL10 are found in immune cells and endothelial cells, our results suggest that chemokines are produced by infiltrating immune cells (26–28). Consistent with $CD8^+$ T-cell infiltration, CXCL9 was found more specifically in tumors of mice receiving IL-13Rα2 DNA vaccination. In contrast, expression of CXCL10 was present in both control and IL-13Rα2-vaccinated tumors, although higher in the latter. This may be because D5α2 tumors may have attracted a basal level of nonspecific $CD8^+$ T cells, inducing more the sensitive CXCL10 chemokine through interaction with CXCR-3 (27).

As we found that the IL-13Rα2 DNA vaccine successfully protected against D5α2 tumor challenge, this vaccine was also assessed in established D5α2 tumor. The use of this vaccine mediated regression of established tumor but complete regression was not observed. The mechanism of therapeutic IL-13Rα2 DNA vaccination involved infiltration of $CD4^+$ and $CD8^+$ T cells, suggesting a role similar to that in prophylactic vaccine protection.

Our current results may be extrapolated to CTL in clinical settings, and it is possible that both $CD4^+$ and $CD8^+$ T cells and antibody against the IL-13Rα2 chain might be induced by IL-13Rα2 vaccination as observed in this animal study. However, to achieve an optimal therapeutic effect in the clinic, it may be useful to vaccinate with the IL-13Rα2 DNA vaccine followed by a second vaccination with an IL-13Rα2 peptide vaccine or virus vector expressing the IL-13Rα2 gene. These approaches have been shown to be more effective than DNA vaccination followed by DNA vaccination (1, 3, 41–43). Alternatively, the IL-13Rα2 protein mixed with adjuvants would be useful, in addition to the DNA vaccine, to enhance the immune response as a prime-boost strategy. These types of preclinical studies will be needed to translate our observations to the clinic for the treatment of patients with glioblastoma, head and neck cancer, kidney cancer, ovarian cancer, and Kaposi's sarcoma.

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Characterization of a Novel Human Tumor Antigen Interleukin-13 Receptor α2 Chain

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