

Specific Recognition and Killing of Glioblastoma Multiforme by Interleukin 13-Zetakine Redirected Cytolytic T Cells

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ABSTRACT

The interleukin (IL) 13 receptor $\alpha 2$ (IL13R $\alpha 2$) is a glioma-restricted cell-surface epitope not otherwise detected within the central nervous system. Here, we describe a novel approach for targeting glioblastoma multiforme (GBM) with IL13R $\alpha 2$ -specific cytolytic T cells (CTLs) by their genetic modification to express a membrane-tethered IL13 cytokine chimeric T-cell antigen receptor, or zetakine. Our prototype zetakine incorporates an IL13 E13Y mutein for selective binding to IL13R $\alpha 2$. Human IL13-zetakine⁺CD8⁺ CTL transfectants display IL13R $\alpha 2$ -specific antitumor effector function including tumor cell cytotoxicity, T_C1 cytokine production, and zetakine-regulated autocrine proliferation. The E13Y amino acid substitution of the IL13 mutein of the zetakine endows CTL transfectants with the capacity to discriminate between IL13R $\alpha 2$ ⁺ GBM targets from targets expressing IL13R $\alpha 1$. *In vivo*, the adoptive transfer of IL13-zetakine⁺CD8⁺ CTL clones results in the regression of established human glioblastoma orthotopic xenografts. Pilot clinical trials have been initiated to evaluate the feasibility and safety of local-regional delivery of autologous IL13-zetakine redirected CTL clones in patients with recurrent GBM. Our IL13-zetakine is a prototype of a new class of chimeric immunoreceptors that signal through an engineered immune synapse composed of membrane-tethered cytokine muteins bound to cell-surface cytokine receptors on tumors.

INTRODUCTION

The malignant phenotype of transformed cells is determined in part by deregulated signaling cascades originating from receptor complexes on the plasma membrane (1). Additionally, tumors frequently disable signaling pathways that serve to enforce cellular homeostatic control mechanisms by expressing decoy receptors (2). Thus, a variety of cell-surface receptors that have a direct bearing on tumorigenicity constitute potential targets for therapeutic intervention. The utilization of receptor ligands for the targeted delivery of oncologic therapeutics has, to date, primarily focused on the development of cytokine cytotoxic fusion proteins and ligand cell attachment chimeras for redirecting viral vector tumor cell tropism (3–6).

Interleukin-13 $\alpha 2$ cytokine receptor (IL13R $\alpha 2$) is expressed on >80% of high-grade gliomas, including glioblastoma multiforme (GBM), and is not otherwise present in the central nervous system (CNS; refs. 7–9). IL-13 cytotoxins have been described that display potent *in vitro* and *in vivo* antitumor activity and are under investigation in phase I/II clinical trials (10–12). Second-generation cytotoxins are being developed that employ IL-13 muteins with enhanced

selective binding to IL13R $\alpha 2$, as compared with the more ubiquitous immune system IL13R $\alpha 1$ /IL4R α receptor complex (13, 14). The E13Y IL-13 mutein displays a 50-fold higher affinity for IL-13 $\alpha 2$ and a 5-fold lower affinity for IL13R $\alpha 1$ /IL4R α as compared with wild-type IL-13 (13). IL13R $\alpha 2$ -selective IL-13 cytotoxins may have significant advantages for targeting glioma, because the shared IL13 $\alpha 1$ /IL4R α receptor complex is expressed within the CNS (15).

Here, we show that a prototype zetakine immunoreceptor with a membrane-tethered IL13 E13Y mutein can redirect the antigen specificity and antitumor effector mechanisms of cytolytic T cells (CTLs) to IL13R $\alpha 2$ ⁺ gliomas. We hypothesize that IL13R $\alpha 2$ -specific CTL clones, delivered in a locoregional fashion to the CNS, will be uniquely suited to eradicate a dispersed population of invasive glioma tumor cells.

MATERIALS AND METHODS

Construction of an IL13-Zetakine cDNA. The IL13-zetakine cDNA was assembled by splice-overlap PCR. The IL-13 (E13Y) cDNA was *de novo* synthesized by PCR with primers designed based on the IL-13 mRNA sequence reported by Smirnov *et al.* (16) and then modified to have a 5' human GM-CSFR α leader sequence and a 3' human immunoglobulin G4-CD4 transmembrane-cytoplasmic CD3- ζ cDNA sequence. The completed construct contains a Kozak consensus ribosome-binding sequence and flanking *Xba*I and *Not*I restriction sites. This construct was ligated into the multiple cloning site of the mammalian plasmid expression vector pMG (Invivogen, San Diego, CA) under the control of the human Elongation Factor 1 α promoter (EF1p). This plasmid (referred to as the IL13-zetakine/HyTK-pMG) was further modified to coexpress the hygromycin phosphotransferase-HSV thymidine kinase (HyTK) selection/suicide fusion gene under the control of the cytomegalovirus immediate-early promoter (17). Plasmid vector was linearized by *Pac*I (New England BioLabs, Beverly, MA) digestion, and the linearized DNA was purified and resuspended in sterile water at 2 mg/mL for T-cell electroporation.

Cell Lines and Cultures. The human T-cell line Jurkat; human glioma lines SN-B19, U87, U251, U138, and T98; human neuroblastoma lines Be2 and 10HTB; and human lymphoma line Daudi were obtained from American Type Culture Collection (Rockville, MD). The tumorigenic line of U251, designated U251T, was the kind gift of Dr. Waldemar Debinski (Wake Forest University, Winston-Salem, NC). Normal skin fibroblasts were derived from healthy donor skin biopsy and established in culture as an adherent line. Lymphoblastoid lines were established from Epstein-Barr virus-infected human peripheral blood mononuclear cells in the presence of cyclosporine (Bedford Laboratories, Bedford, OH) per standard practice (18).

T-cell lines and clones were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum (FCS), 25 mmol/L HEPES-BSS, and 2 mmol/L L-glutamine supplemented with 50 U/mL rIL-2 (Chiron, Emeryville, CA). Lymphoblastoid lines and Daudi cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 25 mmol/L HEPES-BSS, and 2 mmol/L L-glutamine. Adherent cell lines SN-B19, U251, U138, Be2, 10HTB, and fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 25 mmol/L HEPES-BSS, and 2 mmol/L L-glutamine.

Isolation, Activation, and Electroporation of Human T Cells. Peripheral blood mononuclear cells were isolated by density gradient centrifugation over Ficoll-Paque (Pharmacia Biotech, Piscataway, NJ) from heparinized peripheral blood obtained from consented healthy donors participating on a City of Hope National Medical Center Institutional Review Board-approved protocol. The

Received 2/20/04; revised 9/9/04; accepted 10/8/04.

Grant support: National Institutes of Health grant RO1 CA103959, 5P30 CA33572-20, the Brain Tumor Society, the Michael Hoefflin Foundation, the Ronald Flax Memorial Pediatric Cancer Research Fund, the Amy Philips Charitable Foundation, and the Estelle, Abe, and Marjorie Sanders California Foundation. M. Jensen was a recipient of the Stop Cancer Foundation Career Development Award and the Lauren's Run Fellowship. C. Brown was a Leukemia and Lymphoma Society of America Research Fellow.

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activation of T cells with OKT3 followed by electroporation, and subsequent cloning and propagation in hygromycin/rhIL-2 has been described previously (19). Jurkat T cells were electroporated when in log-phase growth and cloned in limiting dilution 48 hours later in cytotoxic concentrations of hygromycin (Stratagene, La Jolla, CA) as described previously (20).

Flow Cytometric Analysis of Jurkat and Primary T-Cell Transfectants. Cell-surface expression of the IL13-zetakine construct was evaluated by staining a phycoerythrin-conjugated antihuman IL-13 monoclonal antibody (Becton Dickinson, San Jose, CA) and an IL13R α 2/hIgG1 chimera (R&D Systems, Minneapolis, MN) detected with a fluorescein isothiocyanate-conjugated antihuman IgG1 monoclonal antibody (Sigma, St. Louis, MO). The cell-surface phenotype of primary human T-cell transfectants was assayed with fluorescein isothiocyanate-conjugated anti-CD4, anti-CD8, and anti-TCR α/β monoclonal antibodies (Becton Dickinson).

Western Blot for Chimeric Receptor Expression. Whole-cell lysates of bulk T-cell transfectants were processed for protein electrophoresis, transfer, and blotting, according to standard methods as described previously (20). Blots were probed with an antihuman CD3- ζ mAb 1D3 (BD PharMingen, San Diego, CA). Tunicamycin (Sigma) at 5 μ g/mL was added to T-cell cultures as indicated 24 hours before harvesting cells.

Chromium Release Assay for Redirected Tumor Cell Killing. The cytolytic activity of T-cell effectors was determined by 4-hour chromium release assay as described previously (21). Effectors cells were used 10 to 14 days after stimulation with OKT3 and irradiated feeder cells. Percent specific lysis values were derived from the average of triplicate wells with SEM values.

Analysis of Cytokine Production. T-cell responder cells (2×10^6) were cocultured in 24-well tissue culture plates with irradiated (8,000 rads) stimulator cells (2×10^5) in 2 mL of culture medium. Rat antihuman IL-13 monoclonal antibody (BD PharMingen) and rhIL-13 (R&D Systems) were used as blocking agents by addition to T-cell responders and glioma stimulators, respectively, at concentrations of 1, 10, 100, and 1000 ng/mL 30 minutes before plating. After a 72-hour incubation, culture supernatants were assayed by cytokine-specific ELISA per the manufacturer's instructions (R&D Systems) and by cytometric bead array using the Human Th1/Th2 Cytokine kit per the manufacturer's instructions (BD PharMingen).

T-Cell Proliferative Assays. T-cell responders (1×10^5) were cocultured with Mitomycin-C treated stimulators (0.1×10^5) in quadruplicate in 96-well U-bottomed plates. After a 72-hour coculture, wells were pulsed with 1 μ Ci/well [methyl- 3 H]thymidine. Twelve hours later, DNA was harvested onto fiberglass filter paper, and 3 HTdR incorporation was quantitated on a liquid

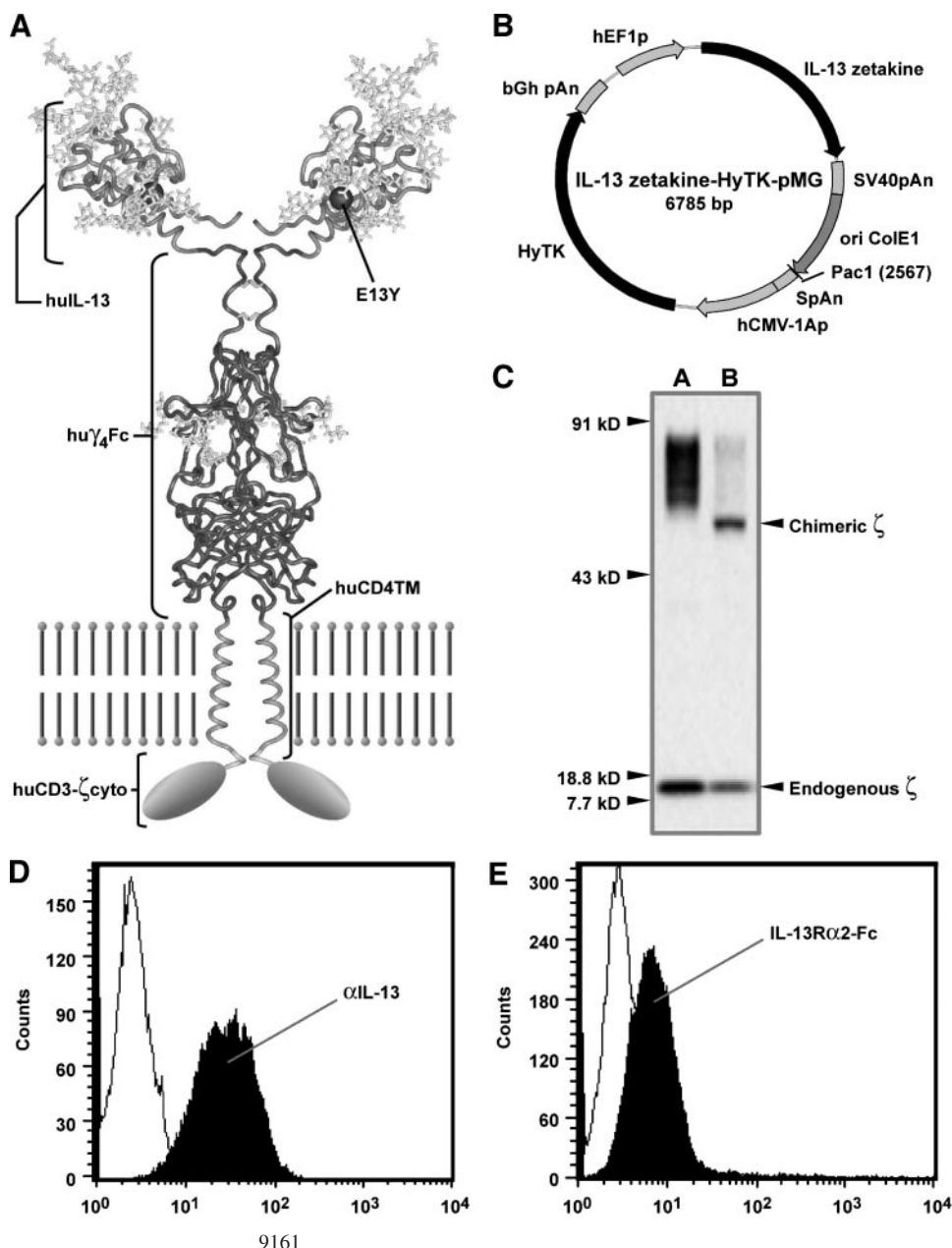


Fig. 1. IL13-zetakine expression, cell-surface trafficking, and IL13R α 2 binding. *A*, schematic representation of the IL-13 zetakine chimeric immunoreceptor consisting of an extracellular human hinge γ_4 Fc membrane-tethered IL-13 E13Y mutant, huCD4TM, and the intracellular huCD3- ζ cytoplasmic tail. *B*, diagram of the expression vector IL13-zetakine/HyTK-pMG. *C*, Western blot analysis of IL-13 zetakine expression of reduced whole-cell lysates derived from Jurkat T-cell transfectants probed with the mouse antihuman CD3 ζ -specific monoclonal antibody. *Lane A*, no cell pretreatment with tunicamycin; *Lane B*, tunicamycin pretreated cells. *D*, flow cytometric detection of cell-surface IL-13 zetakine on cloned human T-cell transfectants. *E*, binding of soluble IL13R α 2/hIgG1 chimera to zetakine-expressing T-cell transfectants detected with fluorescein isothiocyanate-conjugated antihuman IgG1 monoclonal antibody.

scintillation β -counter. Blocking anti-IL13 was added to glioma stimulators at concentrations of 1, 10, and 100 ng/mL 30 minutes before plating.

Orthotopic Glioma Xenograft Model. Male 6- to 8-week-old NOD-*scid* mice were anesthetized with an intraperitoneal injection of 132 mg/kg ketamine and 8.8 mg/kg xylazine. Mice were shaved on the head above the nape of the neck, scrubbed with betadine/alcohol, and immobilized in a Cunningham Mouse/Neonatal Rat Adaptor stereotactic apparatus restraint (Stoelting, Wood Dale, IL). A 5-mm skin incision was made along the sagittal suture and a burr hole drilled into the skull, 2 mm lateral and 0.5 mm anterior to the bregma. Animals were then placed in the E15600 Lab Standard Stereotaxic Instrument (Stoelting) for injection of cells using a 30-gauge 5- μ L Hamilton syringe mounted on a motorized stereotactic injector, over 3 to 5 minutes. U87-ffLucZeo/IL2⁺ tumor cells (2×10^5 cells/mouse) suspended in 2 μ L of phenol-free deficient RPMI (Irvine Scientific, Irvine, CA) were injected as follows: 1 μ L injected at a 2.5 mm depth from the dura, and 1 μ L injected at a 2.25 mm depth from the dura. Animals were treated with 10×10^5 T cells delivered in 2 μ L to the tumor coordinates 5 days after tumor inoculation. Burr holes were sealed with bone-wax and the incision closed with Nexaband glue. To aid post-surgical recovery and prevent hypothermia, animals received a subcutaneous injection of 0.1 mg/kg Buprenex and were placed on a heating pad until a righting reflex was observed.

Biophotonic Tumor Imaging. Animals received injections of 4.29 mg per mouse of freshly prepared luciferin substrate suspended in PBS. Mice were then anesthetized with Isoflurane (1.5 L oxygen + 4% isoflurane per minute) in an induction chamber. After induction of deep anesthesia, mice were imaged using the Xenogen IVIS *In Vivo* Imaging System (Xenogen, Palo Alto, CA) 14 minutes post intraperitoneal injection of luciferin at a 1-minute acquisition time in small binning mode. Luciferase activity was analyzed through Living Image Software from Xenogen to quantify tumor region flux (photons per second).

Brain Histology/Immunohistochemistry. Mice were euthanized via CO₂ inhalation at day +100 post-tumor injection. Mice were transcardially perfused with 4% paraformaldehyde. The brain tissue was post-fixed overnight and embedded in paraffin, and histology was performed on 10- μ m serial horizontal sections. Sections were incubated for 3 hours at 58°C. Tissue sections were stained by a standard hematoxylin and eosin technique. Sister sections were processed by a heat-induced retrieval technique using citric buffer at pH 6 and then stained with antimouse glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (Ventana Medical Systems, Tucson, AZ) using an autostainer (TechMate, Ft. Collins, CO). Sections were processed using Dako EnVision+ systems (peroxidase) for the blocking steps as well as the secondary antibody staining as per the manufacturer's instructions (DakoCytomation,

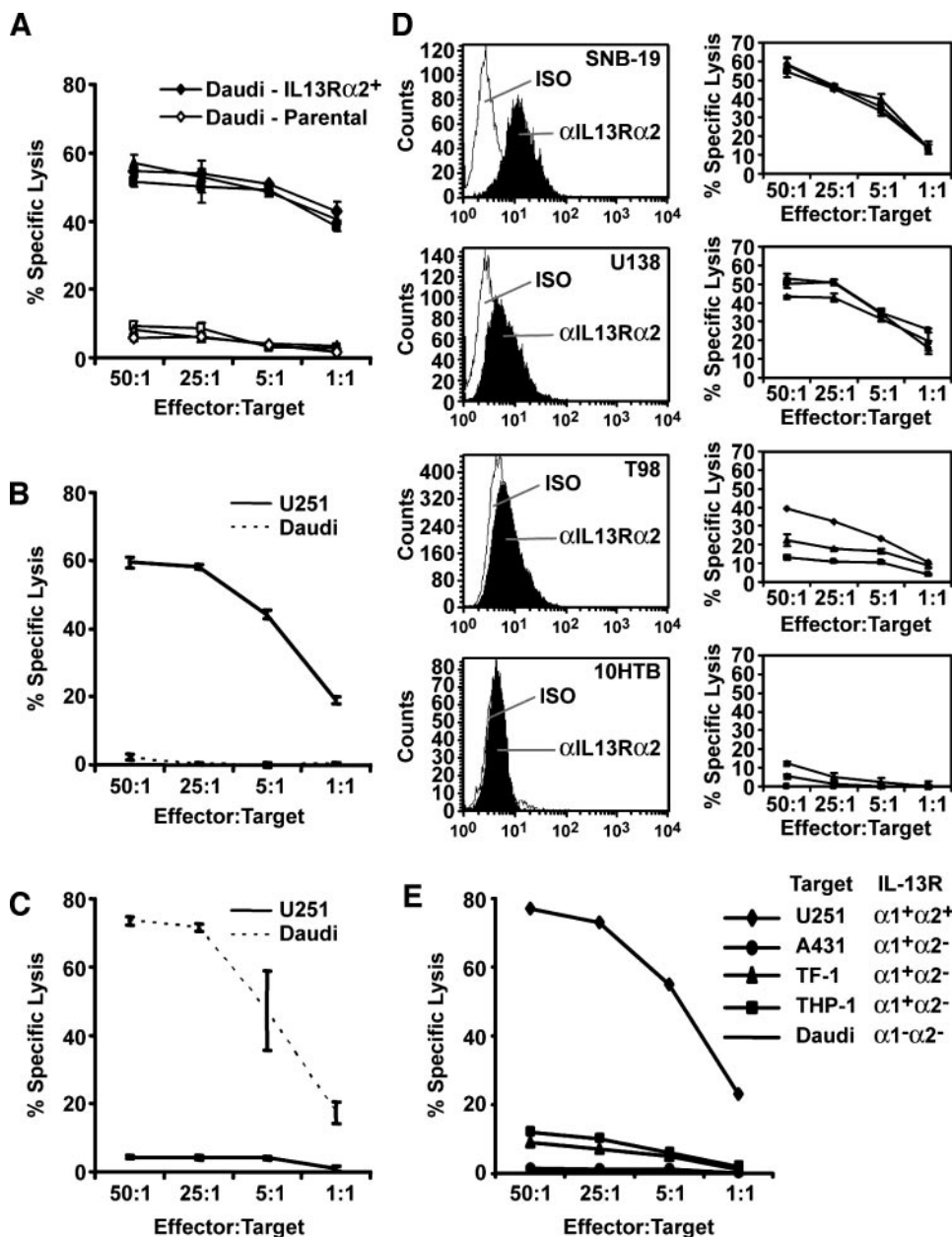


Fig. 2. IL13-zetakine⁺ CD8⁺ CTL clones specifically lyse IL13R α 2⁺ target cells. A, lytic activity of cloned IL13-zetakine⁺ CD8⁺ CTL effectors against IL13R α 2⁻ Daudi cells (open symbols) or IL13R α 2⁺ Daudi transfectants (filled symbols). B, IL13zetakine⁺ CTL effectors lyse U251 glioma (IL-13R α 2⁺/CD20⁻) but not Daudi lymphoma (IL-13R α 2⁻/CD20⁺) targets. C, CD20-specific CTL effectors lyse Daudi lymphoma (IL-13R α 2⁻/CD20⁺) targets but not U251 glioma (IL-13R α 2⁺/CD20⁻). D, correlation of IL13R α 2 expression by flow cytometry (left column) with cytolytic activity of IL13-zetakine redirected CTL (right column). E, IL13-zetakine redirected clones discriminate between IL-13R α 1⁺ α 2⁻ targets (A431, TF-1, and THP-1) versus IL13R α 2⁺ U251.

Carpinteria, CA). Diaminobenzidine (DakoCytomation) was used as a chromogen. Sections were counterstained in 50% Mayer's hematoxylin for 1 minute.

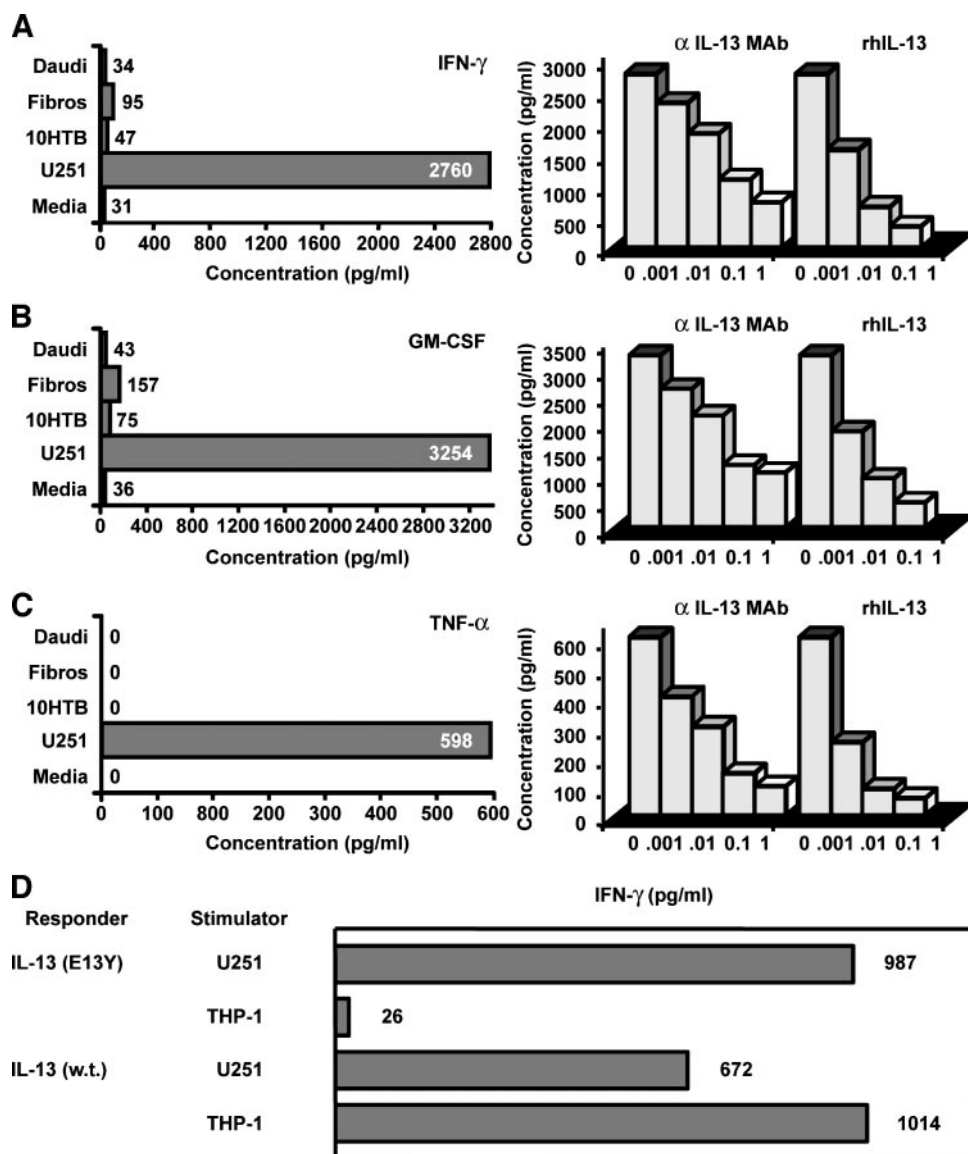
RESULTS

Expression of the Interleukin 13-Zetakine by T-Cell Transfectants. The IL13-zetakine consists of an extracellular IL-13 E13Y mutein-human IgG4 hinge-Fc chimera fused to human cytoplasmic CD3- ζ via the transmembrane domain of human CD4 (Fig. 1A). The mammalian plasmid expression vector designated IL13-zetakine/HyTK-pMG directs the transcription of the IL13-zetakine from a modified human Elongation-1 α promoter and the selection/suicide HyTK fusion protein from a cytomegalovirus immediate/early promoter (Fig. 1B). After transfection with the IL13-zetakine/HyTK-pMG plasmid, Jurkat T cells were cloned by limiting dilution in cytotoxic concentrations of hygromycin. Drug-resistant clones were assayed for zetakine expression by Western blotting using an anti-human CD3- ζ (cytoplasmic tail) mAb that detects both endogenous CD3- ζ and the chimeric zeta chain. Reduced whole-cell lysates of Jurkat transfectants demonstrate endogenous CD3- ζ (M_r ~16,000 band) as well as the IL13-zetakine, migrating as a diffuse set of bands

consistent with the heavy glycosylation of human IL-13 (Fig. 1C, Lane A). When Jurkat T-cell transfectants were coincubated overnight with tunicamycin, an inhibitor of N-linked glycosylation, then processed for Western analysis, the IL13-zetakine resolved to a single band having an electrophoretic mobility in accordance with the predicted M_r 54,000 of the zetakine immunoreceptor polypeptide (Fig. 1C, Lane B). We also processed lysates under nonreducing conditions, with and without tunicamycin, and the resulting banding pattern was consistent with the expressed zetakine forming a homodimer through disulfide bonds present in IgG4 heavy chain dimers (data not shown). Trafficking of the zetakine to the cell surface as a type I transmembrane protein was confirmed by binding of a phycoerythrin-conjugated IL13-specific antibody to cloned primary human TCR α/β^+ CD4 $^+$ CD8 $^+$ CTL transfectants (Fig. 1D). Recombinant IL13R α 2-immunoglobulin fusion protein also bound to IL13-zetakine $^+$ transfectants (Fig. 1E), thereby demonstrating that the extracellular membrane-tethered IL-13 E13Y ligand assumes a nominal conformation for binding to IL13R α 2.

Redirected IL13R α 2-Specific Glioma Tumor Cell Cytolysis. The specificity of target recognition of IL13-zetakine $^+$ CTL for redirected IL13R α 2-specific tumor cell cytotoxicity was evaluated using

Fig. 3. IL13-zetakine-regulated cytokine production. Stably transfected IL13-zetakine $^+$ T cells were cocultured with irradiated stimulator cells (Daudi lymphoma cells, fibroblasts, 10HTB neuroblastoma cells, or U251 glioma cells). In the left column, culture supernatants were assayed for IFN- γ (A), GM-CSF (B), and tumor necrosis factor- α (C). In the right column, blocking rat antihuman IL13 antibody and rhIL13 were added to T-cell responders and U251 glioma stimulators and assayed for effect on cytokine production. D. IL13(E13Y)-zetakine $^+$ clone and IL-13 (wild-type) zetakine-expressing clones were cocultured with IL-13R α 1 $^+$ α 2 $^+$ glioma cells (U251) or IL-13R α 1 $^+$ α 2 $^-$ cells (THP-1) and assayed for production of IFN- γ .



4-hour chromium release assays. We demonstrate that expression of a human IL13R α 2 transgene by Daudi lymphoma cells sensitizes these cells for redirected killing by primary human IL13-zetakine⁺ CD8⁺ CTL clones (Fig. 2A). IL13-zetakine⁺ CTL efficiently lysed U251 GBM targets that constitutively express IL13R α 2 with no demonstrable killing of IL13R α 2⁻ Daudi (Fig. 2B). Antiglioma lytic activity was shown to be mediated through the IL13-zetakine because CTLs expressing a CD20-specific scFvFc: ζ chimeric immunoreceptor fail to kill CD20⁻ U251 GBM while having lytic activity against CD20⁺ Daudi targets (Fig. 2C). Next, the relationship between cell-surface density of IL13R α 2, as quantified by flow cytometry, and sensitivity to lysis by IL13-zetakine⁺ CTL clones was assessed (Fig. 2D). Glioma lines expressing disparate cell-surface densities of IL13R α 2 were lysed by CTL effectors, including T98 glioma targets that express as few as 500 IL-13 binding sites per cell. We also examined the capacity of the IL-13 E13Y mutant of the IL13-zetakine to discriminate between IL13R α 2⁺ and IL13R α 1/IL-4R α 1⁺ targets. Consistent with the observed differential affinities of the IL-13 E13Y mutant for these two IL-13 receptor complexes, we did not detect CTL activation for target cell lysis of the IL13R α 1⁺/IL13R α 2⁻ cell lines A431, TF-1, and THP-1 (Fig. 2E). In aggregate, these results demonstrate that the IL13-zetakine chimeric immunoreceptor endows primary human CTL with the capacity for IL13R α 2-specific recognition and killing of glioma tumor cells.

Interleukin 13-Zetakine-Regulated Cytokine Production. To determine whether the IL13-zetakine provides an activation signal for cytokine production upon engagement of T-cell transfectants with GBM, we cocultured cloned IL13-zetakine⁺ CTL responders and glioblastoma (U251), neuroblastoma (10HTB), lymphoma (Daudi), and fibroblast stimulators. We then assayed supernatants from these cocultures for cytokine content by ELISA. IL13-zetakine⁺ CD8⁺ CTL responders produced interferon (IFN)- γ , GM-CSF, and tumor necrosis factor- α when cocultured with IL13R α 2⁺ U251 glioma stimulators but not when the IL-13R α 2⁻ 10HTB, Daudi, and fibroblast cell lines were used as stimulators (Fig. 3, left column). To demonstrate that cytokine production was mediated by zetakine engagement of IL-13R α 2 on tumor stimulators, we assessed the ability of antihuman IL-13 mAb and rhuIL-13 to block this interaction and inhibit CTL activation for cytokine synthesis. Addition of anti-IL13 IgG and rhuIL-13 to cultures containing CTL and U251 resulted in a dose-dependent inhibition of cytokine production (Fig. 3, right column). Consistent with the IL13R α 2 selectivity of target cell recognition observed in the cytotoxicity data set, the IL13R α 1/IL4R α 1⁺ THP-1 cell line failed to activate IL13(E13Y)-zetakine⁺ CTL clones for cytokine production. Conversely, CTL transfectants expressing a wild-type IL13-zetakine were triggered by both IL13R α 2⁺ glioma and IL13R α 1⁺ THP-1 stimulators (Fig. 3D).

Interleukin 13-Zetakine-Regulated Cytolytic T-Cell Autocrine Proliferation. We next evaluated the capacity of the IL13-zetakine to transduce an activation signal capable of inducing cellular proliferation of CTL transfectants. CD8⁺ CTL clones were stimulated with Mitomycin C inactivated IL13R α 2⁺ glioblastoma cells (SN-B19, U138, U251, and U138) and IL13R α 2⁻ neuroblastoma cells (Be2 and 10HTB) or fibroblasts, with or without addition to culture of 5 U/mL rhuIL-2. Proliferative activity, as monitored by [³H]thymidine incorporation, was observed when IL13-zetakine⁺ responders were stimulated with IL-13R α 2⁺ glioma cells (stimulation indexes > 50; Fig. 4A). Proliferation occurred without the requirement for addition to culture of exogenous IL-2. GBM stimulated proliferation required a zetakine signal as evidenced by the inhibition of the proliferative response with the addition of blocking anti-IL13 IgG (Fig. 4B). The IL13-zetakine regulated helper-independent proliferation of these

CTL clones corresponded with their capacity to make IL-2 upon stimulation with GBM (Fig. 4C).

Regression of Glioblastoma Xenografts after the Adoptive Transfer of Interleukin 13-Zetakine⁺ Cytolytic T Cells. The *in vivo* antiglioma activity of adoptively transferred IL13-zetakine⁺ CTL was demonstrated using an orthotopic xenograft model employing established U87 glioblastoma cells stably transfected to express the firefly luciferase-Zeocin resistance fusion protein and human IL2. In this model, 2 \times 10⁵ U87 tumor cells are stereotactically implanted in the forebrain of NOD-scid animals. Five days later when tumors establish themselves and have increased 10-fold in tumor ff-Luc flux from that of day +1 post injection (data not shown), mice were treated by stereotactic delivery of 10 \times 10⁵ IL13-zetakine⁺ CD8⁺ CTL clone 2D7. Complete regression of tumor flux signal was observed within 72 to 96 hours of adoptive transfer, whereas all mice given the same dose of a CTL clone expressing a CD19-specific CAR demonstrated tumor progression. The Kaplan-Meier progression-free survival curves of these two cohorts of animals are displayed in Fig. 5B. Treated mice remained neurologically asymptomatic and upon sacrifice demonstrated a moderate degree of gliosis in brain parenchyma adjacent to the treated tumor bed (Fig. 5C).

DISCUSSION

Antigen-specific CD8⁺ CTLs can survey the CNS parenchyma and kill antigen-positive astrocytes without collateral damage to neurons

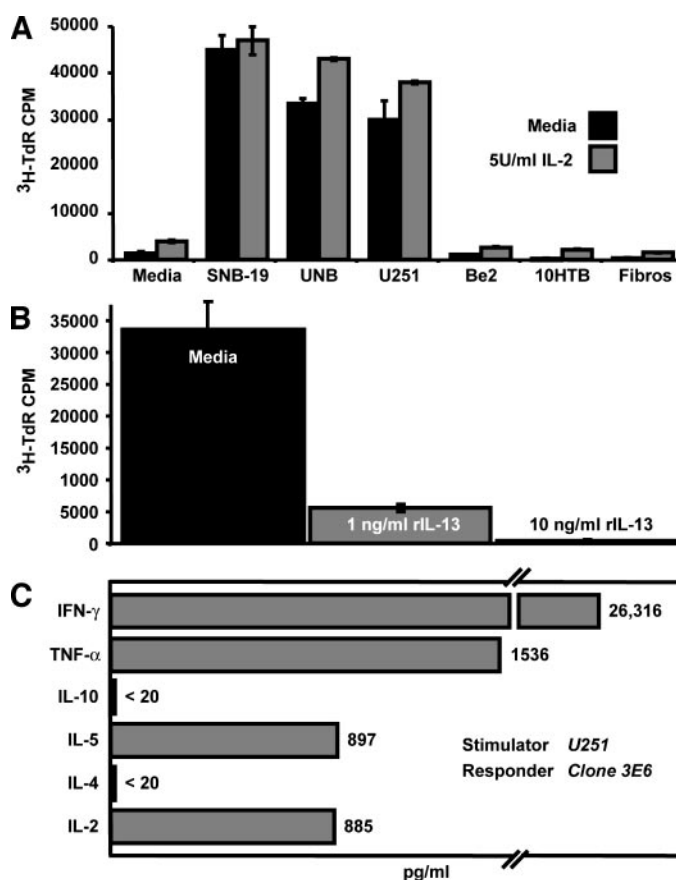


Fig. 4. IL13-zetakine regulated CTL proliferation. A, IL13-zetakine⁺ T-cell responders were cocultured with Mitomycin-C-treated IL13R α 2⁺ glioblastoma (SN-B19, U138, U251, and U138), IL13R α 2⁻ neuroblastoma (Be2 and 10HTB), and fibroblast stimulators in the presence and absence of IL-2 (5 U/mL). After a 48-hour incubation, the cultures were pulsed with [³H]thymidine, and incorporated radioactivity of DNA was measured approximately 12 hours thereafter. B, effect of blocking anti-IL13 added to U138 glioma stimulators on CTL proliferation. C, measurement of IL-2 by cytometric bead array in mixed IL13-zetakine CTL-glioma culture supernatants.

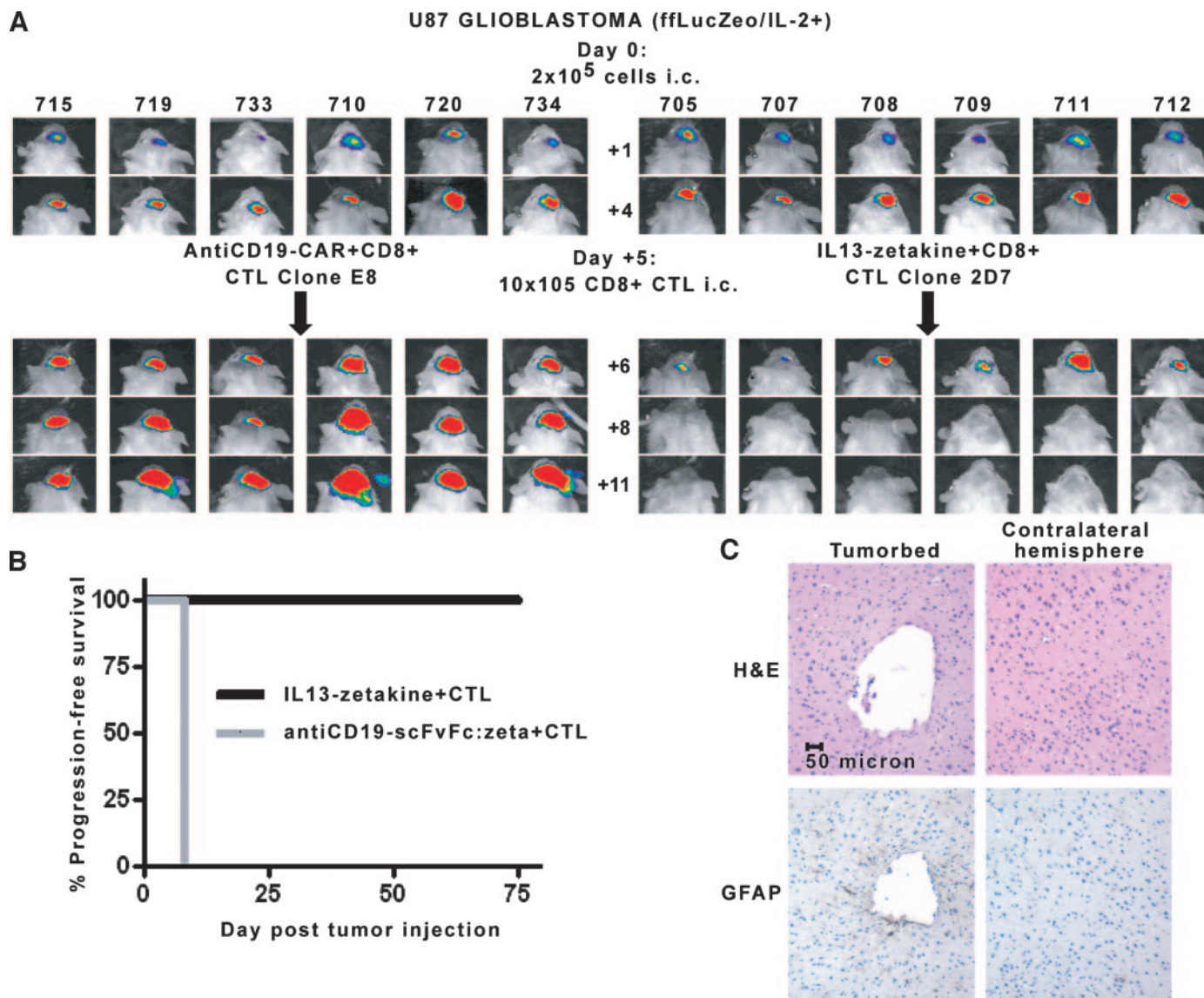


Fig. 5. Regression of established glioblastoma xenografts after adoptive transfer of IL13-zetakine⁺ CTL. A. Firefly luciferase/huIL2-expressing U87 GBM cells (2×10^5) were stereotactically implanted into the right forebrain of NOD-*scid* mice. Five days later, animals received either 10×10^5 IL13-zetakine⁺ CD8⁺ CTL clone 2D7 (right panel) or antiCD19-scFvFc:zeta⁺ CD8⁺ clone E8 (left panel) to the tumor bed. Flux was quantified using the Xenogen Living Image system for a region of interest encompassing the head of the animal after administration of luciferin substrate to anesthetized animals. B. Kaplan-Meier progression-free survival for the two treatment cohorts. C. representative brain histology of the tumor bed and contralateral hemisphere of a IL13-zetakine treated mouse 100 days after initial tumor grafting. Hematoxylin and eosin and anti-GFAP immunohistochemically processed sister sections are presented.

and oligodendrocytes or myelin (22). Syngeneic orthotopic rodent adoptive therapy models have been used to study the antiglioma activity of adoptively transferred immunologic effector cell populations and have provided proof-of-principle that tumor-specific CTLs can mediate glioma regression (23). Additional syngeneic models in rat have further delineated the antitumor potency of tumor-specific CTLs administered locoregionally (24). Antigen-nonspecific LAK cells fail to eradicate tumor in most of these models (25). These studies demonstrate that CTLs have the capacity to migrate into brain parenchyma, whereas LAK cells remain sequestered at the site of local administration. No obvious neurologic toxicities were observed in these model systems, in particular the initiation of autoimmune encephalitis.

The translation of recent conceptual and technological advances in the fields of molecular immunology and tumor immunobiology to adoptive therapy of human malignant glioma is hampered by the paucity of genetically defined antigens expressed by these tumors to

which T-cell responses can be generated, the challenges of isolating antigen-specific effector cells from this patient population, and the low levels of HLA class I molecules expressed on the surface of these tumor cells *in vivo* (26). To overcome these challenges, our group has focused on T-cell genetic engineering strategies for targeting the glioma-restricted cell-surface antigen IL13R α 2. The genetic modification of T cells to express zetakine chimeric receptors for redirected tumor recognition obviates both the requirement for pre-existing immune responses to these tumors and the requirement that tumor cells express HLA Class I molecules.

Our data demonstrate that primary human CTL transfectants expressing a chimeric immunoreceptor using membrane-tethered IL-13 E13Y mutein are triggered for antitumor effector functioning upon engagement of IL13R α 2 on gliomas. Furthermore, the IL-13 E13Y mutein of this zetakine construct equipped CTL to distinguish between targets expressing the tumor-restricted IL13R α 2 from targets that express the IL13R α 1/IL4R α receptor complex. In addition to

lysing GMB targets, IL13-zetakine redirected CTLs are activated for T_H1 cytokine production and helper-independent proliferation. Our observation that GBM stimulators activate CTL to make autocrine IL-2 is consistent with a second costimulatory signal being supplied to CTL by these tumor cells and, of interest, may be a consequence of expression by these tumors of NKG2D ligands, including MICA (27).⁵ The antitumor activity of CTL clones was verified using an orthotopic model system in which established rapidly growing human glioblastoma xenografts regressed after intracranial adoptive transfer of an IL13-zetakine⁺ CD8⁺ CTL clone. Although a Food and Drug Administration–authorized pilot study (BB IND#10109) has been initiated to study the feasibility and tolerability of delivering autologous IL13-zetakine⁺ CD8⁺ CTL clones to tumor resection cavities in the setting of locally recurrent malignant glioma, preclinical development of additional T-cell genetic engineering strategies are currently focusing on equipping CTL for enhanced survival and retention of function in the glioma microenvironment.

Of significant import to the optimization of the epitope specificity and immunobiology of zetakine immunoreceptor signaling, as exemplified by our IL13-zetakine, is the ability to modulate the binding affinity and target specificity of these chimeras through the introduction of amino acid substitutions in the zetakine targeting domain. The additional adaptation of ligand-based chimeras to recruit costimulatory receptors, such as CD28 and 4-1BB, into the immune synapse between T cells and tumor cells is under development by our group and might be used in conjunction with zetakine CD3-ζ. The application of these technologies for the treatment of malignant glioma will serve as a prototype system for defining the optimized formulation of molecularly engineered zetakine redirected T cells in cancer immunotherapy.

ACKNOWLEDGMENTS

We thank C. Wright, W. Chang, A. Naranjo, J. Wagner, L. Bruins, T. Kahute, E. Lin, A. Castro, and B. Aguilar for technical assistance; J. Mayo and K. Carlson for assistance with manuscript preparation; and P. Greenberg and S. Riddell for helpful discussions.

REFERENCES

1. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70.
2. Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999;17:2941–53.
3. Pastan I, Kreitman RJ. Immunotoxins in cancer therapy. *Curr Opin Investig Drugs* 2002;3:1089–91.
4. Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol* 1996;70:6839–46.

5. Zhou G, Ye GJ, Debinski W, Roizman B. Engineered herpes simplex virus 1 is dependent on IL13Ralpha 2 receptor for cell entry and independent of glycoprotein D receptor interaction. *Proc Natl Acad Sci USA* 2002;99:15124–9.
6. Debinski W. Recombinant cytotoxins specific for cancer cells. *Ann NY Acad Sci* 1999;886:297–9.
7. Murata T, Obiri NI, Debinski W, Puri RK. Structure of IL-13 receptor: analysis of subunit composition in cancer and immune cells. *Biochem Biophys Res Commun* 1997;238:90–4.
8. Mintz A, Gibo DM, Slagle-Webb B, Christensen ND, Debinski W. IL-13Ralpha2 is a glioma-restricted receptor for interleukin-13. *Neoplasia* 2002;4:388–99.
9. Husain SR, Puri RK. Interleukin-13 receptor-directed cytotoxin for malignant glioma therapy: from bench to bedside. *J Neurooncol* 2003;65:37–48.
10. Joshi BH, Husain SR, Puri RK. Preclinical studies with IL-13PE38QQR for therapy of malignant glioma. *Drug News Perspect* 2000;13:599–605.
11. Debinski W, Obiri NI, Powers SK, Pastan I, Puri RK. Human glioma cells overexpress receptors for interleukin 13 and are extremely sensitive to a novel chimeric protein composed of interleukin 13 and pseudomonas exotoxin. *Clin Cancer Res* 1995;1:1253–8.
12. Barth S. hIL-13-PE38QQR. *Neopharm Curr Opin Investig Drugs* 2001;2:1309–13.
13. Debinski W, Gibo DM, Obiri NI, Kealisher A, Puri RK. Novel anti-brain tumor cytotoxins specific for cancer cells. *Nat Biotechnol* 1998;16:449–53.
14. Nash KT, Thompson JP, Debinski W. Molecular targeting of malignant gliomas with novel multiply-mutated interleukin 13-based cytotoxins. *Crit Rev Oncol Hematol* 2001;39:87–98.
15. Debinski W, Gibo DM. Molecular expression analysis of restrictive receptor for interleukin 13, a brain tumor-associated cancer/testis antigen. *Mol Med* 2000;6:440–9.
16. Smirnov DV, Smirnova MG, Korobko VG, Frolova EI. Tandem arrangement of human genes for interleukin-4 and interleukin-13: resemblance in their organization. *Gene* 1995;155:277–81.
17. Lupton SD, Brunton LL, Kalberg VA, Overell RW. Dominant positive and negative selection using a hygromycin phosphotransferase-thymidine kinase fusion gene. *Mol Cell Biol* 1991;11:3374–8.
18. Pelloquin F, Lamelin JP, Lenoir GM. Human B lymphocytes immortalization by Epstein-Barr virus in the presence of cyclosporin A. *In Vitro Cell Dev Biol* 1986;22:689–94.
19. Jensen MC, Clarke P, Tan G, et al. Human T lymphocyte genetic modification with naked DNA. *Mol Ther* 2000;1:49–55.
20. Jensen M, Tan G, Forman S, Wu AM, Raubitschek A. CD20 is a molecular target for scFvFc:zeta receptor redirected T cells: implications for cellular immunotherapy of CD20+ malignancy. *Biol Blood Marrow Transplant* 1998;4:75–83.
21. Cooper LJ, Topp MS, Serrano LM, et al. T-cell clones can be rendered specific for CD19: toward the selective augmentation of the graft-versus-B-lineage leukemia effect. *Blood* 2003;101:1637–44.
22. Cabarros J, Bauer J, Piaggio E, Liblau R, Lassmann H. Effective and selective immune surveillance of the brain by MHC class I-restricted cytotoxic T lymphocytes. *Eur J Immunol* 2003;33:1174–82.
23. Yamasaki T, Handa H, Yamashita J, Watanabe Y, Namba Y, Hanaoka M. Specific adoptive immunotherapy with tumor-specific cytotoxic T-lymphocyte clone for murine malignant gliomas. *Cancer Res* 1984;44:1776–83.
24. Holladay FP, Heitz T, Chen YL, Chiga M, Wood GW. Successful treatment of a malignant rat glioma with cytotoxic T lymphocytes. *Neurosurgery* 1992;31:528–33.
25. Holladay FP, Heitz T, Wood GW. Antitumor activity against established intracerebral gliomas exhibited by cytotoxic T lymphocytes, but not by lymphokine-activated killer cells. *J Neurosurg* 1992;77:757–62.
26. Lampson LA, Hickey WF. Monoclonal antibody analysis of MHC expression in human brain biopsies: tissue ranging from “histologically normal” to that showing different levels of glial tumor involvement. *J Immunol* 1986;136:4054–62.
27. Raullet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* 2003;3:781–90.

⁵ M.C. Jensen, unpublished data.

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Specific Recognition and Killing of Glioblastoma Multiforme by Interleukin 13-Zetakine Redirected Cytolytic T Cells

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Cancer Res 2004;64:9160-9166.

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