

Stem-like Tumor-Initiating Cells Isolated from IL13R α 2 Expressing Gliomas Are Targeted and Killed by IL13-Zetakine-Redirected T Cells

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Abstract

Purpose: To evaluate IL13R α 2 as an immunotherapeutic target for eliminating glioma stem-like cancer initiating cells (GSC) of high-grade gliomas, with particular focus on the potential of genetically engineered IL13R α 2-specific primary human CD8⁺ CTLs (IL13-zetakine⁺ CTL) to target this therapeutically resistant glioma subpopulation.

Experimental Design: A panel of low-passage GSC tumor sphere (TS) and serum-differentiated glioma lines were expanded from patient glioblastoma specimens. These glioblastoma lines were evaluated for expression of IL13R α 2 and for susceptibility to IL13-zetakine⁺ CTL-mediated killing *in vitro* and *in vivo*.

Results: We observed that although glioma IL13R α 2 expression varies between patients, for IL13R α 2^{pos} cases this antigen was detected on both GSCs and more differentiated tumor cell populations. IL13-zetakine⁺ CTL were capable of efficient recognition and killing of both IL13R α 2^{pos} GSCs and IL13R α 2^{pos} differentiated cells *in vitro*, as well as eliminating glioma-initiating activity in an orthotopic mouse tumor model. Furthermore, intracranial administration of IL13-zetakine⁺ CTL displayed robust antitumor activity against established IL13R α 2^{pos} GSC TS-initiated orthotopic tumors in mice.

Conclusions: Within IL13R α 2 expressing high-grade gliomas, this receptor is expressed by GSCs and differentiated tumor populations, rendering both targetable by IL13-zetakine⁺ CTLs. Thus, our results support the potential usefulness of IL13R α 2-directed immunotherapeutic approaches for eradicating therapeutically resistant GSC populations. *Clin Cancer Res*; 18(8); 2199–209. ©2012 AACR.

Introduction

The prognosis of patients with high-grade gliomas remains grim despite improvements in standard therapies including surgery, radiation, and chemotherapy. The deadly nature of this disease is, in part, attributed to therapeutically resistant subpopulations of glioma stem-like cancer-initiating cells (GSC) that display many characteristics of normal stem cells, including expression of stem cell markers and the potential for sustained self-renewal (1, 2). GSCs exhibit potent tumor-initiating potential and are hypothe-

sized to seed disease recurrence (3–5) because of their resistance to conventional chemotherapy and radiation regimens.

Cellular immunotherapies have the potential to target and eliminate this therapeutically resistant GSC population (6). Recent promising data have shown that tumor stem cells are susceptible to both CTL and natural killer perforin-dependent killing mechanisms (7–11). Furthermore, GSCs and differentiated tumor cells express equivalent levels of markers involved in immunologic synapse formation, and CD8⁺ CTL can eliminate cell populations capable of tumor engraftment (9). Although challenges remain in addressing the confounding effects of tumor immunosuppressive networks, particularly as they relate to specific strategies that may be employed by tumor stem cells (12–14), the intrinsic ability of immune cells to recognize and kill tumor stem cells is sufficiently powerful to warrant efforts to overcome these obstacles.

IL-13 receptor α 2 (IL13R α 2) is a cell-surface receptor that has received significant attention from brain tumor therapy because it is overexpressed by a subset of high-grade gliomas, but not expressed at significant levels by normal brain tissue (15–18). Our group has developed an IL13R α 2-targeting immunotherapy platform for high-grade gliomas by genetically modifying T cells to express an IL13R α 2-

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Translational Relevance

Patient-to-patient heterogeneity of malignant gliomas, together with the presence of the glioma stem-like cancer initiating cells (GSC), thought to be responsible for disease progression and recurrence, have proven to be barriers to disease eradication. In light of these challenges, our study focused on the usefulness of IL13R α 2-directed therapies for targeting the tumorigenic GSC population of high-grade gliomas. Our findings provide evidence that for the subset of patients with IL13R α 2-positive tumors, immune-based therapies targeting IL13R α 2 have the potential to eliminate both the GSCs and bulk differentiated populations.

specific chimeric antigen receptor (CAR) termed IL13-zetakine (19). This CAR recognizes IL13R α 2 via a membrane-tethered IL13 ligand and initiates cytolytic killing via an intracellular CD3-zeta T-cell activating domain. It also incorporates a point mutation in the IL13 ligand domain (E13Y) for preferential binding to IL13R α 2 and reduced affinity to the more ubiquitously expressed IL13 receptor, IL13R α 1 (20). Previous studies using U87 and other long-established cell lines show that engineered IL13-zetakine⁺T cells exhibit potent MHC-independent, IL13R α 2-specific anti-glioma cytolytic activity, and induce regression of orthotopic xenografts *in vivo* (19). Realization of the potential of therapies targeting IL13R α 2 for the treatment of malignant glioma, some of which are in clinical trial (21–24), will hinge, in part, on their ability to eradicate malignant stem/initiating tumor cell subpopulations. Here we assess the expression of IL13R α 2 on stem-like versus differentiated glioma populations and evaluate the relative susceptibility of glioma cell populations of varying differentiation status to IL13-zetakine⁺ CTL-mediated killing.

Materials and Methods

Cell lines

Glioma specimens, graded according to World Health Organization–established guidelines (Supplementary Table S1), were obtained from patients in accordance with Institutional Review Board (IRB)-approved protocols. Tumor specimens were deidentified and given unique patient brain tumor (PBT) numbers. For one tumor obtained from a patient who participated in our adoptive T-cell therapy clinical trial, it was also referred to by its unique patient number (UPN) to indicate the patient from whom this tumor was derived (PBT015-UPN033). Short-term tumor sphere (TS) cultures of GSCs and adherent lines were expanded and cultured as previously described (9). In some cases tumor explants were expanded by heterotopic subcutaneous passaging in mice before growth and characterization in culture (9), and in such cases the subcutaneous passage number is reported after the PBT number (for instance, PBT009-2 was subcutaneously passaged twice,

and PBT003-4 and PBT017-4 were subcutaneously passaged 4 times). Differentiated lines (DIF) were generated by growing TSs in Sato's Media [DMEM:F12, 2 mmol/L, L-glutamine, 25 mmol/L HEPES, and 7% fetal calf serum (FCS)] for 7 to 14 days. Adherent lines (ADH) were generated by growing freshly dispersed tumor cells directly in Sato's media and used for assays between passages 7 to 30.

Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors or patients in accordance with IRB-approved protocols. The generation of IL13-zetakine⁺ (HD003 IL13-zetakine⁺ clone 2D7) and CD19R⁺ (HD181 CD19R⁺ clone E8) CD8⁺ T-cell lines has been described previously (19, 25). CD8⁺ T-cell lines derived from a patient who participated in our adoptive T-cell therapy clinical trial is referred to by its UPN designation (UPN033). Healthy donor T cell lines were deidentified and designated unique healthy donor (HD) numbers. T-cell clones were cultured as described (19), and all assays were carried out between days 10 through 14 of the stimulation cycle. Lymphoblastoid lines (LCL) were established from Epstein–Barr virus–infected human PBMCs (19). LCL-OKT3 cell lines were engineered to express a membrane-bound OKT3 single-chain antibody (OKT3scFv) as described previously (26).

Flow cytometric analysis

Cell-surface expression of IL13R α 2 was detected using goat polyclonal anti-IL13R α 2 (AF146) (R&D Systems), followed by murine anti-goat fluorescein isothiocyanate (Jackson ImmunoResearch) (Supplementary Table S2). Cell-surface expression of CD133 was detected as described (9) with phycoerythrin (PE)-conjugated mouse anti-human CD133/1 and anti-human CD133/2 (Miltenyi Biotec) (Supplementary Table S2). Percent of immunoreactive cells was calculated using the subtraction method via FCS Express version 3 software (De Novo Software).

Western blot analysis

Western blots were probed with goat polyclonal anti-IL13R α 2 (AF146) and anti-Olig2 (R&D Systems); rabbit polyclonal anti-Actin (Rockland); and mouse monoclonal anti- β -III Tubulin (Millipore), anti-CD133 (Miltenyi), and anti-GFAP (Sigma-Aldrich) antibodies as per the manufacturers' instructions (Supplementary Table S2). Western blots were imaged on the Odyssey Infrared Imaging System (LI-COR), and band intensities were quantified using Odyssey v2.0 software (LI-COR).

Immunofluorescence and immunohistochemistry

TSs were plated on poly-L-ornithine (Sigma) and mouse laminin (BD Biosciences) coated chamber slides to support adherent growth (27). When required, cells were grown for 7 to 10 days in 10% FCS-containing media to promote astrocytic differentiation (GFAP immunoreactive), or 0.5% FCS with 5 μ mol/L forskolin (Sigma) to promote neuronal differentiation (β -III Tubulin immunoreactive). Cells were stained with primary antibodies anti-SOX2 (Abcam), anti-Nestin (Millipore), anti-GFAP (Sigma), and anti- β III

Tubulin (Millipore) (Supplementary Table S2), followed by anti-mouse or anti-rabbit Alexa-488 (Invitrogen), and costained with 4',6-diamidino-2-phenylindole (DAPI) mounting solution (Vector Laboratories, Inc.). Cells were visualized using an AX70 fluorescent microscope (Olympus), and exposure settings for each antibody were kept constant between growth conditions.

Glioblastoma IL13R α 2 immunohistochemistry (IHC) was done on 5- μ m sections of formalin-fixed paraffin-embedded specimens, treated with EnVision FLEX Target Retrieval Solution Low pH (Dako) in a PT Link pretreatment module (Dako) for antigen retrieval. Sections were then blocked with serum-free protein block (Dako), incubated with 3.33 μ g/mL goat polyclonal anti-IL13R α 2 (AF146), and detected using rabbit anti-goat (Dako) and the EnVision FLEX/HRP system (Dako). All staining was done using the DAKO polymer automated system.

For mouse brain IHC, brains were harvested, fixed and embedded in paraffin as described previously (19). Horizontal brain sections (10 μ m) were deparaffinized, underwent citrate-based antigen retrieval, and stained with anti-human Nestin (Millipore), anti-SOX2 (R&D Systems), anti-Olig2 (R&D Systems), or anti-GFAP (Dako) (Supplementary Table S2) followed by detection using the EnVision kit (Vector Laboratories) with 50% hematoxylin counterstain.

qPCR

To quantify *IL13R α 2* mRNA levels, RNA samples were isolated from cells using the RNeasy Kit (Qiagen), and cDNA was prepared using the Superscript First Strand Synthesis System for reverse transcription PCR (RT-PCR) (Invitrogen). Real-time PCR analysis was done using Sybr-Green (Qiagen) with specific primer pairs for *IL13R α 2* (GenBank; NM_000640.2) spanning exon 1–2 and exons 6–7 and *β -actin* (internal PCR control) (Supplementary Table S3). Fold-difference in *IL13R α 2* mRNA levels compared with the U251T calibrator line was determined using the Livak method (28).

Cytotoxicity and cytokine assays

Chromium release assays (CRA) were carried out as previously described (29). Cytokine production was measured by coculturing T cells with tumor at a 10:1 effector:target (E:T) ratio. In blocking experiments, anti-IL13 antibody (BD Biosciences) and recombinant human IL-13 (Peprotech) were added at the indicated concentrations to responders and stimulators, respectively, 30 minutes before plating. After 20- to 24-hour incubation, supernatants were harvested and assayed using Luminex multiplex bead technology (Upstate), and output data were analyzed via the Bio-Plex Manager 4.0 (BioRad).

Tumor xenografts and tumor volume determination

Mice were maintained under pathogen-free conditions, and all procedures were done with 6- to 8-week-old non-obese diabetic/severe combined immunodeficient (NOD-*scid*) or NOD/*scid*/IL2R $^{-/-}$ (NSG) mice as approved by the COH Institute Animal Care and Use Committee. Intracra-

nial tumor xenografts were prepared as previously described (19). No difference in tumor engraftment between NOD-*scid* and NSG mouse strains has been observed.

Fixed and paraffin-embedded brains were serially sectioned at 10- μ m intervals over the entire extent of the tumor. In most cases, every 20th section was stained with a human-specific nestin antibody (any human cell will be tumor) and visualized with 3,3'-diaminobenzidine (DAB). These sections were scanned at high resolution (Chromavision ACIS II). Brain slice images were aligned and DAB $^{+}$ pixels in each brain slice were segmented and counted using Reconstruct software (<http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm>; ref. 30). In some cases, segmentation was based on color and cell density in hematoxylin and eosin-stained sections. In all cases pixel area was extrapolated over the 200- μ m slice interval and total volumes computed accordingly.

Results

Incidence of glioma-associated IL13R α 2 expression is independent of tumor cell differentiation status

Our group has generated a panel of GSC lines from patient glioblastoma specimens grown as TSs in serum-free media supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF) (9). These GSC TS lines were derived either from freshly dispersed human biopsy specimens or after heterotopic subcutaneous passaging of patient biopsy tissue in immunocompromised mice (9, 31). The expanded GSC TS lines exhibited stem cell-like characteristics: formation of secondary TSs as an indication of *in vitro* self-renewal potential (Fig. 1A), expression of stem cell markers such as CD133, SOX2 and Nestin (Fig. 1B and C), differentiation to express lineage-specific markers such as GFAP and β -III tubulin (Fig. 1C), and formation of orthotopic intracranial tumors in immunocompromised mice (see also ref. 9; Supplementary Table S4).

This resource of patient-derived GSCs allowed us to assess expression of IL13R α 2 on stem-like TS and serum-differentiated (DIF) glioma lines. For these studies, we compared IL13R α 2 protein and mRNA levels by Western, flow cytometry, and quantitative RT-PCR (qRT-PCR; Fig. 2). The specificity of receptor recognition in these assays was confirmed by detection of this receptor on known IL13R α 2 pos glioma lines U251T and U87 (19, 32), but not on the IL13R α 2 neg T98 glioma and Daudi lymphoma cell lines (refs. 19, 32; Fig. 2A–C). It is important to note that IL13R α 2-specific commercial antibodies display significant differences in glioma IL13R α 2 detection. We find that clone B-D13 (Cell Sciences) and GW22455A (GenWay) do not recognize an antigen on gliomas that is consistent with either *IL13R α 2* mRNA expression or functional recognition of IL13R α 2 by IL13-zetakine $^{+}$ CTL (Supplementary Fig. S1; Figs. 2 and 4; refs. 19, 32). These findings raise questions about previously published reports which used these antibodies, particularly the commonly used anti-IL13R α 2 B-D13 antibody (Cell Sciences), to assess the frequency of IL13R α 2 expression on gliomas (18, 33). This inconsistency

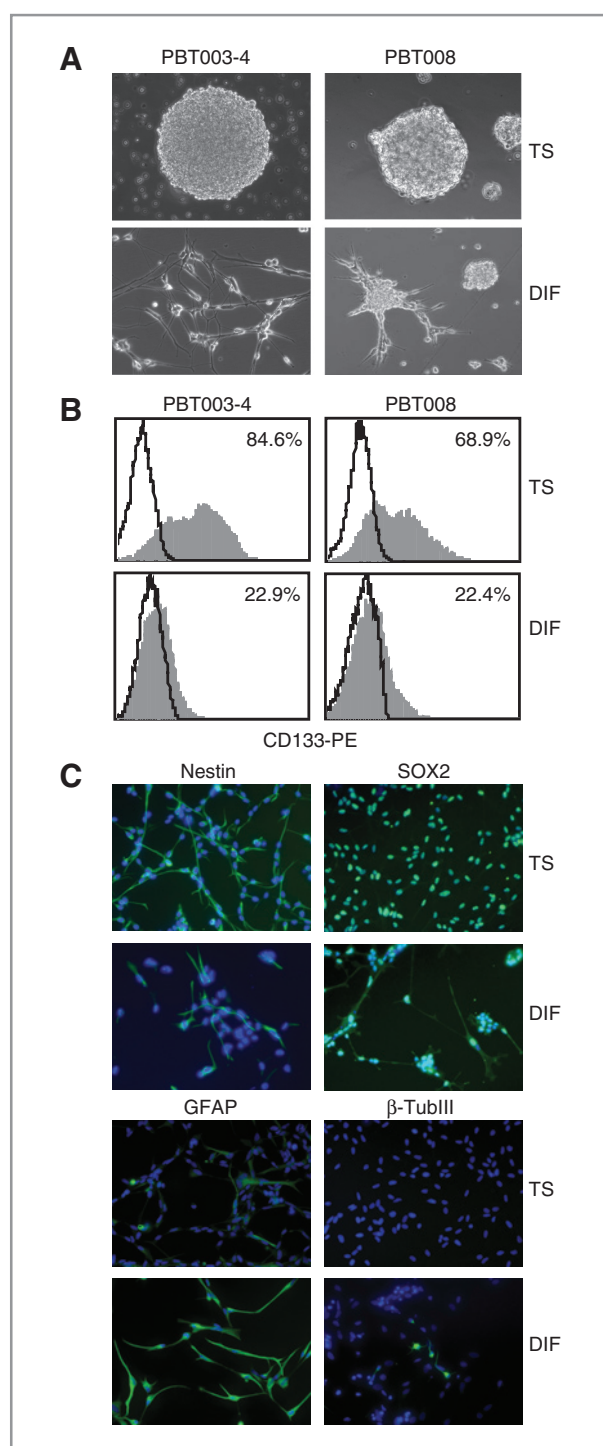


Figure 1. Characterization of TS and serum-differentiated cells isolated from primary high-grade glioma specimens. **A**, representative images of TS cells grown in serum-free stem cell medium (TS, top) and after transfer to differentiating FCS-containing medium (DIF, bottom). **B**, flow cytometry of TS and DIF cells stained with anti-CD133 (gray) or isotype control antibody (solid lines). Percentage of CD133^{pos} cells is indicated. **C**, immunofluorescence of PBT003-4 TS and DIF cells stained for stem cell markers nestin and SOX2 (green, top), and lineage-specific differentiation markers GFAP and β -III tubulin (green, bottom). Cell nuclei were stained blue with DAPI.

may also underlie the lack of correlation between IL13R α 2 mRNA and protein expression for primary glioma biopsy specimens when the anti-IL13R α 2 B-D13 antibody (Cell Sciences) was used for IHC (34). We have therefore used the goat polyclonal anti-IL13R α 2 AF146 antibody (R&D Systems) for all experiments, as it is the only antibody tested that shows a consistent correlation between IL13R α 2 protein levels and mRNA expression [Western and flow cytometry vs. qRT-PCR; Fig. 2A–C, and Supplementary Fig. S1].

We next evaluated IL13R α 2 on stem-like TS lines and found that expression was highly variable between tumor specimens, with a subset of TSs (i.e., PBT015-UPN033, PBT017-4, and PBT030) expressing significant levels of IL13R α 2 (>70% positive by flow cytometry), and approximately half of the tumor lines (i.e., PBT003-4, PBT008, and PBT009-2) not expressing detectable levels of this receptor by either flow cytometry or Western blot (Fig. 2D and E). IL13R α 2 transcript levels, as measured by qRT-PCR, correlated with the observed IL13R α 2 protein expression (Fig. 2C). Our observations are thus in agreement with previous studies showing that the IL13R α 2 receptor is expressed by a subset of patient glioblastoma tumor samples (16, 34).

An expanded normal fetal brain neural stem cell line did not express IL13R α 2 as assessed by flow cytometry and qRT-PCR (Fig. 2C and D). This observation suggested that the IL13R α 2 receptor is not expressed by normal neural stem cells and is in concordance with previous reports that this receptor is not expressed at significant levels in normal brain tissue (15–17, 35).

Significantly, we found that IL13R α 2 is expressed independently of glioma differentiation status. As exemplified by the IL13R α 2^{pos} lines (PBT015-UPN033, PBT017-4, and PBT030), receptor expression is maintained on the stem-like TS (CD133^{pos} Olig2^{pos}), the 7- to 14-day serum-differentiated (GFAP^{pos}; DIF), and the long-term serum-expanded cell populations (ADH; Fig. 2D and E). A small decrease in IL13R α 2 levels was consistently observed following short-term differentiation, but not after long-term growth in serum-containing media (comparing DIF vs. ADH), possibly due to the withdrawal of EGF mitogen (36). Reciprocally, IL13R α 2^{neg} primary lines (PBT003-4, PBT008, and PBT009-2) do not upregulate IL13R α 2^{neg} upon differentiation and growth in serum-containing media (Fig. 2D and E). We concluded that although IL13R α 2 expression by high-grade gliomas is heterogeneous between different tumor samples, its expression on any given low-passage glioma line is independent of cell differentiation status. Thus, these data suggested that, for gliomas expressing IL13R α 2, therapeutic strategies targeting this receptor could potentially eliminate the stem-like brain tumor subpopulation. These studies also indicated the presence of glioma stem-like tumor-initiating populations that do not express IL13R α 2, as detected for the IL13R α 2^{neg} tumors.

To assess the extent to which expression of IL13R α 2 on expanded primary cell lines correlates with the originating patient tumor, IL13R α 2 IHC was done on paraffin-embedded patient tumor tissue. As shown in Fig. 3, patient tumors

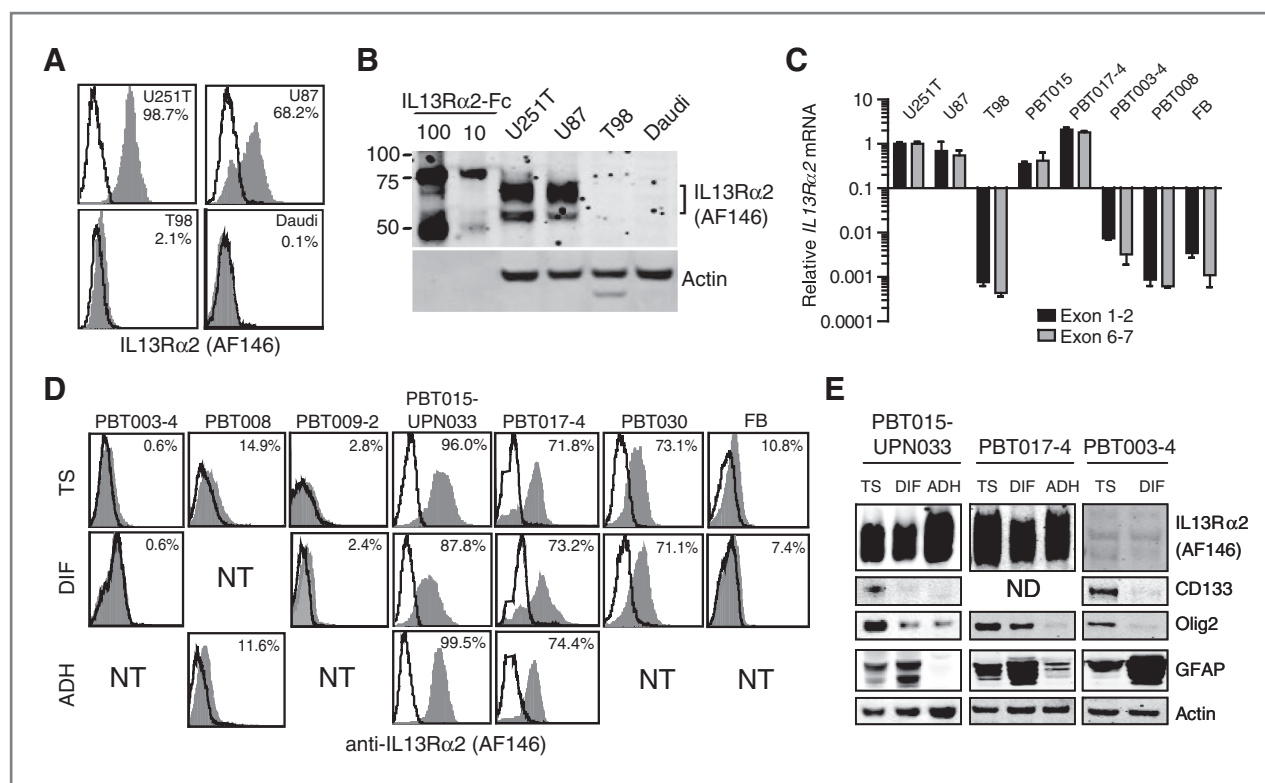


Figure 2. IL13R α 2 expression on GSC and differentiated glioma cell lines. **A**, flow cytometric detection of IL13R α 2 on established glioma cell lines U251T, U87 and T98. Daudi lymphoma is an IL13R α 2^{neg} control cell line. Percentage positive cells are indicated in each histogram. **B**, Western blots detecting IL13R α 2 for established glioma cell lines. Recombinant human IL13R α 2-Fc (10 and 100 ng) shows receptor-specific detection by the antibody. **C**, qRT-PCR analysis depicting *IL13R α 2* mRNA expression by cell lines relative to U251T using primer sets spanning *IL13R α 2* exons 1–2 and 6–7. Data were normalized to *actin*. **D**, TS, serum-differentiated (DIF) and serum-expanded (ADH) cells analyzed by flow cytometry for expression of IL13R α 2 (grey histograms); solid lines are secondary antibody alone. NT, not tested. **E**, Western blots detecting IL13R α 2, CD133, Olig2, GFAP, and α -actin for TS, DIF, and ADH cell lines. ND, not detected.

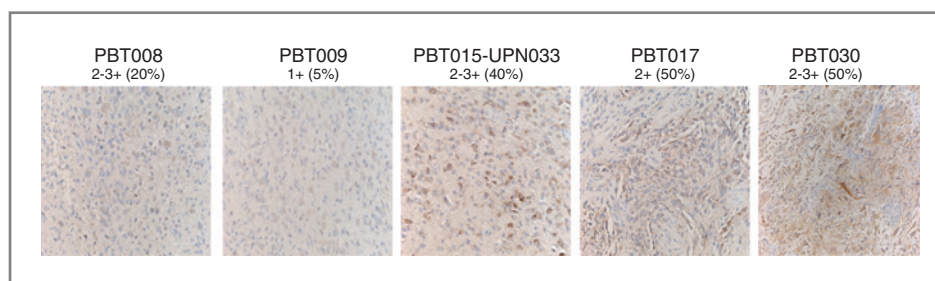
with the highest overall IL13R α 2 expression levels ($\geq 40\%$ of cells positive) as detected by IHC (PBT015-UPN033, PBT017, and PBT030), generated both TS and DIF/ADH lines with correspondingly high IL13R α 2 expression (compare with Fig. 2D and E). Likewise, patient tumors with low IL13R α 2 levels ($\leq 20\%$ positive cells) gave rise to cell lines that lacked receptor expression (PBT008 and PBT009). Importantly, these findings indicated that expression levels of IL13R α 2 on expanded primary glioma cell lines are consistent with its expression by the originating patient tumor. However, on a cell-by-cell basis, IL13R α 2 expression

levels on glioma cells *in situ* were more variable, as detected by IHC, than those of the corresponding donor-derived TS or ADH cell lines (Fig. 2 vs. Fig. 3; refs. 16, 35). This difference may be explained by the more heterogeneous cellular composition of glioma tumors *in situ*.

IL13R α 2 expression renders GSCs susceptible to IL13-zetakine-mediated T-cell killing

We next addressed whether IL13R α 2^{pos} GSCs and differentiated tumor lines display comparable sensitivity to CAR-engineered CTL-mediated killing. For these studies, primary

Figure 3. IL13R α 2 expression on primary patient-derived glioma specimens. IHC detection of IL13R α 2 on paraffin-embedded patient tumor tissue. Sections were scored blindly by a neuropathologist for staining intensity (0 not detected; 1+ low; 2+ moderate; 3+ high), and percentages of positive cells are indicated.



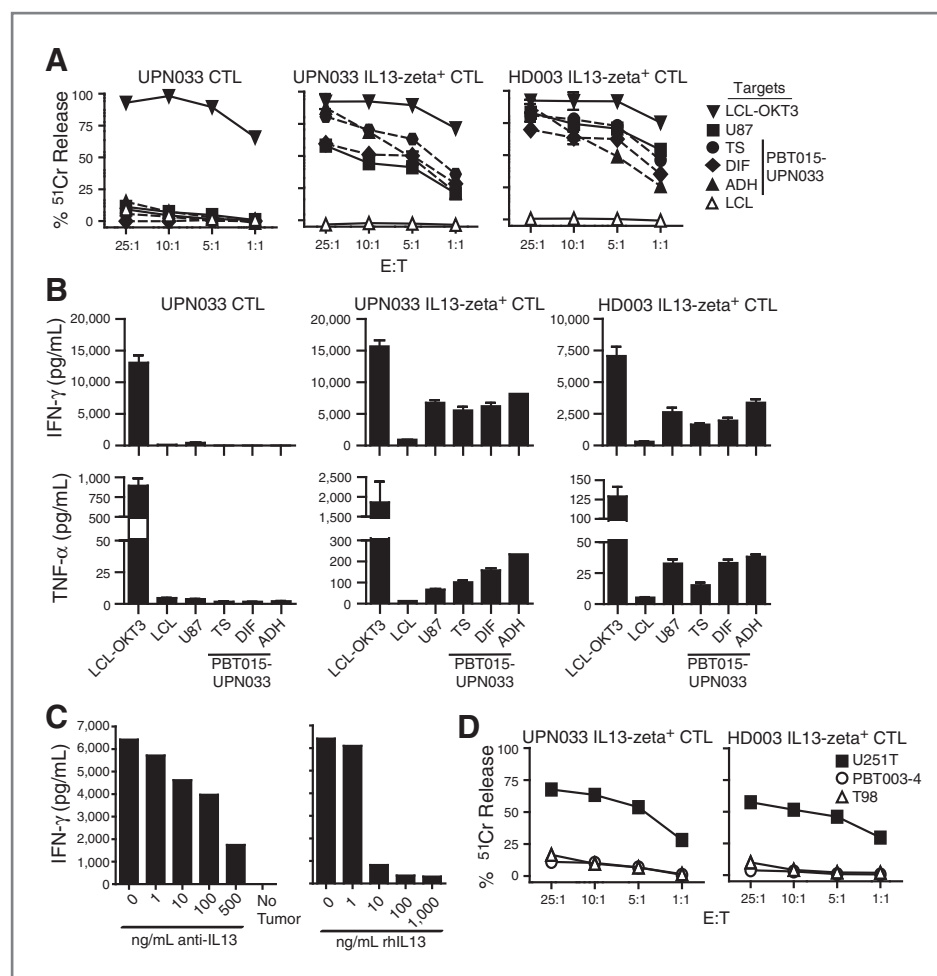


Figure 4. IL13-zetakine⁺ CTL kill with comparable potency IL13R α 2^{pos} GSCs, differentiated cells, and established glioma cell lines. A, CRA measuring the lysis of IL13R α 2^{pos} U87 glioma cells or PBT015-UPN033-derived TS, 7-day serum-differentiated (DIF), or serum-expanded (p15; ADH) cells at increasing E:T ratios. The IL13R α 2^{neg} CD19^{pos} LCL served as an antigen-negative control target, and LCL-OKT3 established the maximum capacity of all CTL lines for cytolytic lysis. Graph titles indicate T-cell effector lines tested, which include the autologous UPN033 parental CD8⁺ CTL bulk line (UPN033 CTL) and CD8⁺ IL13-zetakine⁺ CTL clone 3C12 (UPN033 IL13zeta⁺ CTL), and an allogeneic CD8⁺ IL13-zetakine⁺ CTL clone 2D7 (HD003 IL13zeta⁺ CTL). Mean \pm SD values of 6 wells are depicted. B, IFN- γ and TNF- α produced by the indicated CD8⁺ T-cell lines after overnight coculture with tumor targets described in (A). Mean \pm SD values of 3 replicate measurements from a representative experiment are depicted. C, inhibition of PBT015-UPN033 TS-driven IFN- γ and TNF- α produced by CD8⁺ IL13-zetakine⁺ CTL clone 3C12 as in (B) upon preincubation of the CTL with increasing concentrations of blocking rat anti-human IL13 antibody (left) or PBT015-UPN033 TS target cells with rhIL13 (right). D, CRA measuring lysis of IL13R α 2^{neg} glioma lines PBT003-4 TS and T98 cells versus IL13R α 2^{pos} U251T by the indicated CD8⁺ IL13-zetakine⁺ CTL at increasing E:T ratios. Mean \pm SD values of wells ($n = 6$) are depicted.

human IL13-zetakine⁺ CD8⁺ CTL lines (Supplementary Fig. S2A and B) were evaluated for their ability to kill TS and matched differentiated glioma lines *in vitro*. We observed that autologous (UPN033) or allogeneic (HD003) IL13-zetakine⁺ T-cell clones efficiently lysed IL13R α 2^{pos} PBT015-UPN033 stem-like TSs, as assessed by chromium-release assay (Fig. 4A). For both autologous and allogeneic CTLs, TSs were killed at least as well as matched serum-differentiated (DIF) or serum-expanded (ADH) low-passage primary lines, or established glioma line U87 (average difference in cytotoxicity at all E:T ratios 17% \pm 9% with killing of TS \geq DIF/ADH lines). Lymphoblastic (LCL) targets engineered to express the membrane-bound agonistic antibody scFv-OKT3 (LCL-OKT3), which activates

T cells via engagement of the CD3 complex, served as a reference target denoting the maximal activation potential of a T-cell line. To establish that the capacity of IL13-zetakine CTLs to target and kill IL13R α 2^{pos} GSCs is not specific to a particular TS line, we showed in a similar set of experiments that IL13R α 2^{pos} PBT017-4 and PBT030 TS, DIF, and ADH cells were also recognized and killed independently of differentiation status (Supplementary Fig S3). In all cases, the stem-like TS lines were killed with comparable potency as the serum-differentiated lines.

Following engagement of TS, DIF, ADH, or U87 targets, IL13-zetakine⁺ CTL clones were also activated to produce Tc1 cytokines IFN- γ and TNF- α (Fig. 4B, Supplementary Fig. S4). CTL coculture with PBT015-UPN033 TSs resulted in

slightly less Tc1 cytokine production as compared with serum-differentiated (DIF) cells (average \pm SD decrease in INF- γ for TS vs. ADH and DIF is 42% \pm 7% and 14% \pm 2%, respectively; average decrease in TNF- α for TS vs. ADH and DIF is 58% \pm 2% and 45% \pm 13%, respectively). This decrease in T-cell-mediated INF- γ and TNF- α cytokine release is observed on engagement of all IL13R α 2^{pos} primary glioma lines tested (average 2- to 3-fold decrease) (Supplementary Fig. S4).

Several lines of evidence confirmed that the IL13-zetakine⁺ CTL killing and Tc1 cytokine responses we observed are specific to engagement of IL13R α 2 tumor antigen. First, IL13-zetakine⁺ CTL clones were not activated for killing or cytokine production when coincubated with the IL13R α 2^{neg} PBT003-4 TS and T98 glioma lines or the IL13R α 2^{neg} LCL line (Fig. 4A, B, and D, and data not shown). Second, blocking CAR-IL13R α 2 engagement by either preincubation of PBT015-UPN033 TS targets with recombinant human IL13 (rh-IL13), or IL13-zetakine⁺ CTL with anti-IL13 antibody eliminated cytokine production (Fig. 4C). Third, the parental UPN033 bulk CTL line, which is not engineered to express the IL13-zetakine CAR, displayed negligible levels of killing (<10% ⁵¹Cr-release) and cytokine production (<30 pg/mL INF- γ and <5 pg/mL TNF- α) when challenged with PBT015-UPN033 tumor targets.

These findings confirmed that expression of the IL13R α 2 tumor-associated antigen is necessary for IL13-zetakine⁺ CTL killing (19). Importantly our data establishes that both IL13R α 2^{pos} GSCs and differentiated cells are targeted and killed by IL13-zetakine engineered CTLs, and therefore cells with stem/progenitor-like properties are not intrinsically resistant to CTL-mediated killing.

IL13-zetakine⁺ CTL can ablate the tumor-initiating activity of IL13R α 2^{pos} GSCs *in vivo*

A hallmark characteristic of GSCs is their capacity to initiate *in vivo* tumor formation at low cell numbers. To address the possibility that a minor tumor-initiating subpopulation of IL13R α 2^{pos} cells may evade IL13-zetakine-mediated recognition, an *in vivo* tumor engraftment assay was carried out. This assay establishes that capacity of CTL to eliminate glioma tumor-initiating populations (i.e., the functional definition of GSCs), by providing an *in vivo* read-out of cell populations that resist CTL killing (see Fig. 6 of ref. 9 where antigen-negative tumor populations evaded CTL recognition and initiated tumor). For this study, PBT017-4 TSs were coinjected intracranially into NOD-*scid* mice with either IL13-zetakine⁺ CTL, or a nonspecific CTL line expressing a CD19-specific CAR, or PBS. Because as few as 100 PBT017-4 TS cells can initiate highly invasive tumors in NOD-*scid* mice (9), this assay is expected to detect a minor tumor-initiating population (<0.1%) resistant to IL13-zetakine-mediated CTL killing. We observed that coinjection of IL13-zetakine⁺ CTL, but not PBS or control CD19R⁺ CTL, ablated the engraftment potential of PBT017-4 cells (Fig. 5A). Similarly, IL13-zetakine⁺ CTL, but not PBS or control CD19R⁺ CTL, also ablated the tumor-initiating potential of the established glioma cell line U87 grown

under differentiating conditions (serum-containing media; Fig. 5B). As a control for antigen specificity, we showed that IL13-zetakine⁺ CTL do not ablate the tumor-initiating activity of the IL13R α 2^{neg} line, PBT003-4 TSs (Fig. 5C), consistent with the lack of *in vitro* killing of PBT003-4 when cocultured with IL13-zetakine⁺ CTL (Fig. 4D). This study showed that IL13-zetakine⁺ CTL can eliminate the tumor-initiating activity of both IL13R α 2^{pos} GSCs and differentiated subpopulations.

Regression of established GSC TS-initiated xenografts after adoptive transfer of IL13-zetakine⁺ T cells

We next evaluated the capacity of IL13-zetakine⁺ CTL to induce regression of established GSC TS-initiated tumors. For these studies, we examined the antitumor efficacy of IL13-zetakine⁺ T cells against IL13R α 2^{pos} GSC TS lines PBT030-2 and PBT017-4 (Fig. 2 and Supplementary Fig. S5). To facilitate imaging of tumor growth kinetics, PBT030-2 TSs were engineered by lentiviral transduction to express a fusion protein consisting of EGFP and firefly luciferase (ffLuc; EGFP-ffLuc; ref. 9). EGFP-ffLuc⁺ PBT030-2 or non-engineered PBT017-4 TS cells were orthotopically implanted into the right forebrain of NSG mice. Five days later, after tumor establishment, mice were treated intracranially with 3 infusions of either IL13-zetakine⁺ CD8⁺ CTL clone 2D7 or control CD19R⁺ CD8⁺ CTL clone E8. By comparing ffLuc⁺ tumor flux over time (PBT030-2; Fig. 6A and B) and by quantifying tumor volume 15 days after the last T-cell treatment by IHC (PBT017-4; Fig. 6C and D), we showed that IL13-zetakine⁺ CTL, as compared with control CD19R⁺ CTL, mediate statistically significant anti-glioma activity against both PBT030-2- and PBT017-4-established tumors.

The ability of IL13-zetakine⁺ CTL to regress GSC-initiated established glioblastoma tumors is consistent with our previous observations that IL13-zetakine⁺ CTL can kill IL13R α 2^{pos} TS cells (Fig. 4) and ablate their tumor-initiating activity (Fig. 5). For those mice in which residual tumor was detected following treatment with IL13-zetakine⁺ CTL, we sought to understand the etiology of tumor escape by evaluating the expression of IL13R α 2 in relationship to stemness (Sox2 and Olig2) and differentiation (GFAP) by IHC (day 15 post final T-cell treatment; day 28 post tumor injection). We found that IL13-zetakine⁺ CTL- and control CD19R⁺ CTL-treated tumors express comparable levels of IL13R α 2 (Fig. 6E), suggesting that residual tumors did not evade therapy due to antigen loss. At this 15-day time point, we were not able to detect any persisting CD3⁺ human T cells by IHC (data not shown). Also, we detected comparable levels of SOX2, Olig2, and GFAP (Fig. 6F), suggesting that there is no major difference in the frequency of stem-like versus differentiated cells in the recurrent tumor following IL13-zetakine⁺ CTL-mediated tumor targeting. Although the largest IL13-zetakine⁺ CTL-treated tumor was found in the leptomeninges (Fig. 6D), the significance of this engraftment pattern is not clear as this highly invasive PBT017-4 line (Fig. 5) is known to engraft at this anatomical site. Taken together, these data indicated that exposure to

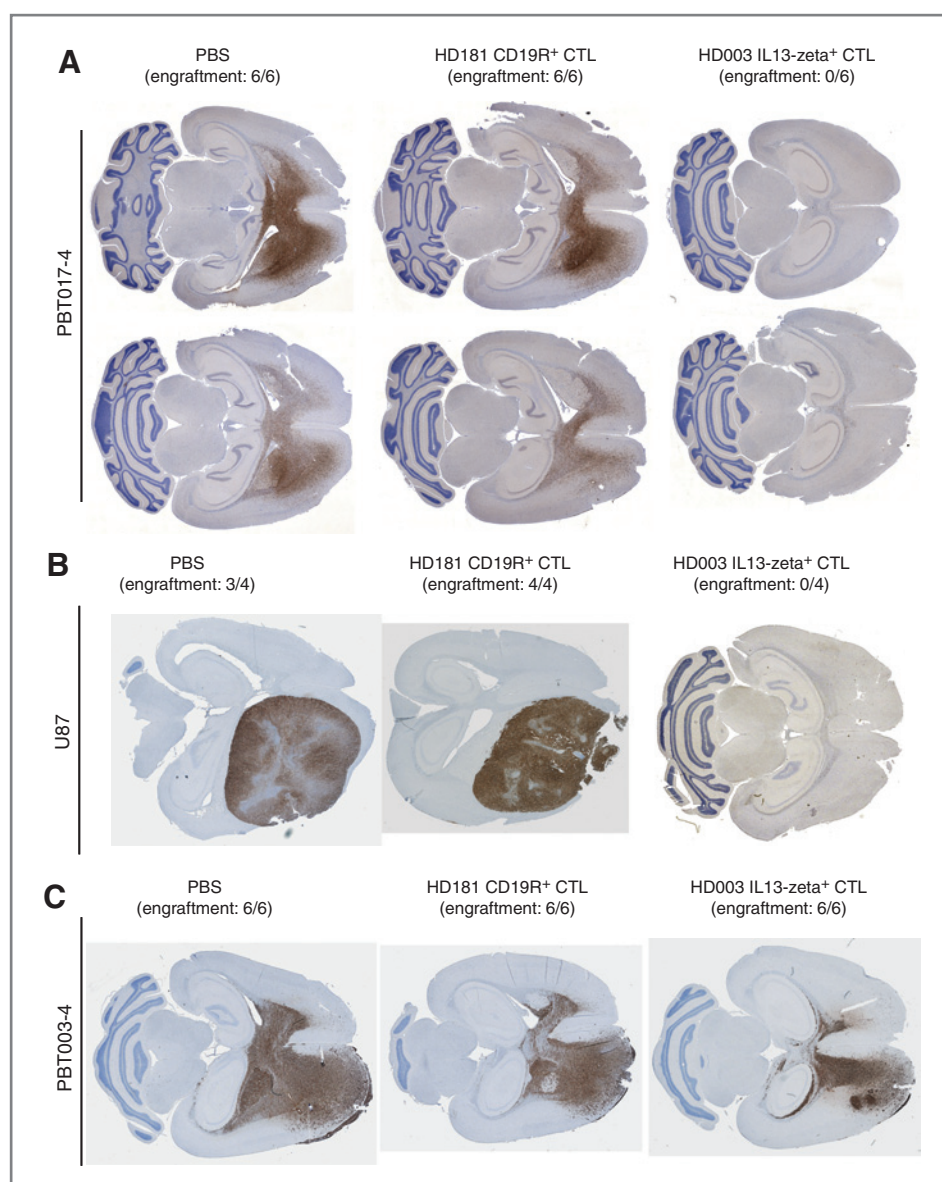


Figure 5. IL13R α 2-specific CTLs ablate the tumor initiation population of IL13R α 2 expressing GSCs. **A**, PBT017-4 TS (2×10^5) were co-injected intracranially with the specified CTL (2×10^6) or PBS into NOD-*scid* mice; $n = 6$ mice per group. Mice were euthanized after 8 weeks, and harvested brains were analyzed by IHC for tumor engraftment using anti-human nestin (number of mice with engrafted tumors per group of 6 is indicated). Representative images from 2 mice per group are depicted (tiled horizontal brain sections). **B**, U87 cells (2×10^5), and **(C)** PBT003-4 TS cells (2×10^5) were co-injected intracranially with CAR⁺ CTLs or PBS in NOD-*scid* mice as described in **(A)**.

IL13-zetakine⁺ CTL has the capacity to reduce tumor growth and tumor burden of established IL13R α 2 expressing tumors. At the same time, these data also suggested that a more potent CAR⁺ T-cell capable of sustained persistence may be required to completely eliminate all IL13R α 2 expressing tumor cells.

Discussion

Although immunotherapy strategies targeting IL13R α 2 for brain tumors are being clinically pursued (21–24), based on its selective expression on malignant versus normal brain tissue (15–18), the power of IL13R α 2-directed therapies will depend, in part, on the ability of these approaches to be effective against the highly tumorigenic glioma cancer stem-like population (3, 4). Our data provide direct evidence that IL13R α 2 expression is not limited

to differentiated glioma cells and that IL13R α 2 targeting can potentially eliminate the refractory GSC component of malignant gliomas. We show that GSC TSs derived from IL13R α 2^{pos} glioma tumors express IL13R α 2 and that its expression renders the IL13R α 2^{pos} GSC population susceptible to IL13-zetakine⁺ CAR-engineered T-cell cytolytic lysis. Importantly, *in vitro* GSC TSs were killed at equivalent potency as matched differentiated glioma lines. We also show that IL13-zetakine⁺ CTL ablate the *in vivo* tumor-initiating activity of IL13R α 2^{pos} GSCs and, further, mediate robust antitumor activity and limit progression of established IL13R α 2^{pos} GSC TS-initiated intracranial tumors in mice.

Although our work and others have shown that GSCs are intrinsically susceptible to effector T-cell killing (7, 8, 9), mechanisms to suppress and/or evade immune function and effector T-cell killing are likely employed by GSCs.

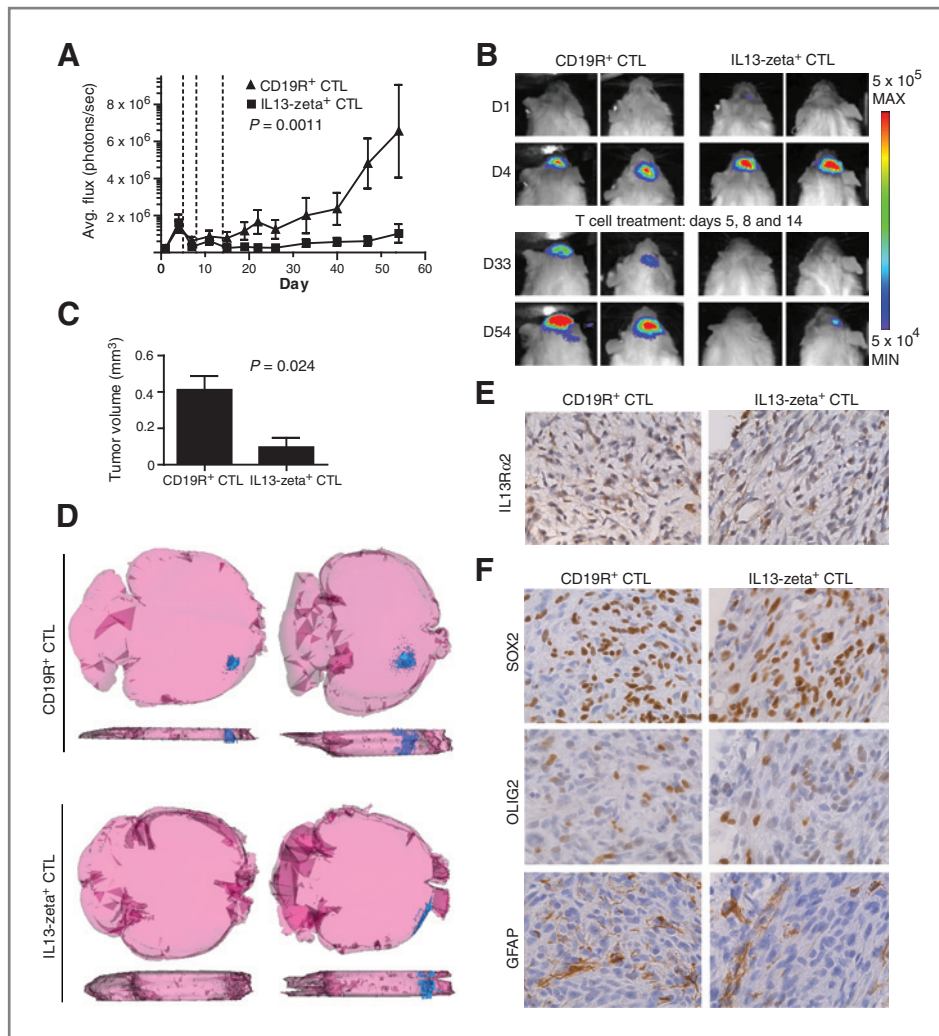


Figure 6. Regression of established GSC-initiated xenografts after adoptive transfer of IL13-zetakine⁺ T cells. **A**, EGFP-ffLuc⁺ PBT030-2 TSs (1×10^5) were stereotactically implanted into the right forebrain of NSG mice. On days 5, 8, and 14 (dotted vertical lines), mice received either 2×10^6 IL13-zetakine⁺ CTL clone 2D7 (HD003 IL13zeta⁺ CTL; $n = 10$) or CD19R⁺ CTL clone E8 (HD181 CD19R⁺ CTL; $n = 8$). Quantification of tumor growth kinetics using Xenogen Living Image to measure average fLuc flux (photons/sec) shows that IL13-zetakine⁺ CTLs induce tumor growth regression when compared with CD19R⁺ CTLs ($P = 0.0011$, 2-way ANOVA with repeated natural log-transformed measures on time, days 15–54). **B**, representative Xenogen images for 2 mice at days 1 and 4 before T-cell administration and at days 33 and 54 post T-cell treatment. **C–F**, xenograft tumors (intracranial) of PBT017-4 (1×10^5) were treated as described in (A) on days 5, 8, and 13. On day 28 (15 days post last T-cell administration) mice brains were harvested and tumor volume was quantified by IHC. **C**, graph depicts the mean tumor volume (mm^3) \pm SE for IL13-zetakine⁺ CTL ($n = 4$) versus CD19R⁺ CTL ($n = 4$) treated tumors ($P = 0.024$, 2-tailed Student *t* test). **D**, the smallest (left) and largest (right) reconstructed tumors (blue pseudocolor) from each group are shown from both the dorsal (top) and sagittal (bottom) views. **E**, anti-IL13R α 2 IHC and (**F**) anti-SOX2, anti-OLIG2, and anti-GFAP IHC on paraffin sections of persisting tumors following adoptive transfer of glioma-specific IL13-zetakine⁺ CTL and control CD19R⁺ CTLs.

Recent studies suggest that GSCs may reduce T-cell activation-dependent proliferation and downmodulate T-cell activation-dependent cytokine production (12, 37). We also find that GSC TSs are less potent at stimulating IL13-zetakine⁺ CTLs for antigen-dependent INF- γ and TNF- α cytokine production (Fig. 4; Supplementary Fig S4). The underlying mechanism of these compromised functionalities and the consequences for antitumor efficacy remain to be resolved. In addition, the malignant stem cell niche is associated with secreted TGF- β and VEGF, as well as activated STAT3, important signals known to inhibit immune

responses and thus may protect GSCs in established tumors from efficient T-cell eradication (12, 38–40). Despite these hurdles, our findings that CD8⁺ CTLs can be engineered to successfully kill the otherwise therapeutically resistant GSC population suggests that efforts to overcome these obstacles are worthwhile.

Clinical trials for recurrent high-grade gliomas using IL13-zetakine–redirected CTLs are in early feasibility/safety stages, and although transient tumor regressions have been observed, sustained regressions are still an unrealized outcome (Jensen; unpublished and ref. 24). This study focused

on the potential barrier of GSCs as a therapeutically resistant reservoir of tumor cells. We provide evidence that disease recurrence following IL13R α 2-directed therapies is not due to the absence of IL13R α 2 expression by all GSC subpopulations, but rather that other mechanisms of escape may also be at play. Furthermore, we show that IL13-zetakine⁺ CTLs can target and kill IL13R α 2^{Pos} GSCs and regress established GSC-initiated tumors. However, for established tumors, IL13R α 2 expressing glioma populations were not completely eliminated, suggesting that a more powerful T-cell product is needed. To this end, CAR-engineered T-cell lines optimized for T-cell persistence and/or serial tumor killing are under development (26, 41, 42).

In addition, clinical targeting of IL13R α 2^{Pos} cells may selectively eliminate not only malignant cells, but also cells within the microenvironment that are involved in immune evasion. Of particular interest, tumor-infiltrating macrophages (CD11b high/Gr-1 intermediate) have been shown to upregulate cell-surface IL13R α 2 expression in the tumor microenvironment and are stimulated by IL-13 to secrete TGF- β , resulting in inhibition of antigen-specific CTL function (43). Blocking induction of IL13R α 2 on tumor-infiltrated macrophages correspondingly inhibits TGF- β production and restores the antitumor activity of CD8⁺ T cells. In these ways, targeting IL13R α 2 expressed by other cells in the tumor microenvironment may also enhance the efficacy of clinically targeting this receptor.

Importantly, the heterogeneity of IL13R α 2 receptor expression by high-grade gliomas, rather than persistence

of refractory GSC populations, is anticipated to be a critical barrier to overcome for clinical responses (Figs. 2 and 3 and refs. 16, 34, 35). A guiding principle now emerging from a variety of clinical trials suggests that immunotherapeutic approaches targeting single antigens will not be sufficient for durable long-term antitumor responses. Our data showing that IL13R α 2 is expressed by a subset of glioma stem-like-initiating populations susceptible to cell immunotherapy highlight the relevance of translational strategies targeting IL13R α 2 for malignant glioma, particularly when incorporated into combinatorial targeting approaches.

Disclosure of Potential Conflicts of Interest

M.C. Jensen is an inventor of licensed patents and equity holder in ZetaRx, Inc., a licensee of these patents. Other authors have no conflicts of interest to disclose.

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