Stem-like Tumor-Initiating Cells Isolated from IL13Rα2 Expressing Gliomas Are Targeted and Killed by IL13-Zetakine–Redirected T Cells

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Abstract

Purpose: To evaluate IL13Rα2 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 IL13Rα2-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 IL13Rα2 and for susceptibility to IL13-zetakine+ CTL-mediated killing in vitro and in vivo.

Results: We observed that although glioma IL13Rα2 expression varies between patients, for IL13Rα2+ 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 IL13Rα2+ GSCs and IL13Rα2+ 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 IL13Rα2+ GSC TS-initiated orthotopic tumors in mice.

Conclusions: Within IL13Rα2 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 IL13Rα2-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 hypothesized 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 α2 (IL13Rα2) 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 IL13Rα2-targeting immunotherapy platform for high-grade gliomas by genetically modifying T cells to express an IL13Rα2-
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 IL13Rα2-directed therapies for targeting the tumorigenic GSC population of high-grade gliomas. Our findings provide evidence that for the subset of patients with IL13Rα2-positive tumors, immune-based therapies targeting IL13Rα2 have the potential to eliminate both the GSCs and bulk differentiated populations.

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 UPN numbers. For one tumor specimen, tumor explants were expanded in a nonhuman primate model. Healthy donor T cell lines were deidentified and designated unique healthy donor (HD) numbers. Cell-surface expression of CD133 was detected as described previously (26). Differentiated lines (DIF) were generated from Epstein–Barr virus–infected human PBMCs (19). LC-L-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 IL13Rα2 was detected using goat polyclonal anti-IL13Rα2 (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-IL13Rα2 (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

Tiss 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 μmol/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-βIII...
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 IL13Rt2 immunohistochemistry (IHC) was done on 5-μm 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 μg/mL goat polyclonal anti-IL13Rt2 (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 μm) 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 IL13Rα2 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 IL13Rα2 (GenBank: NM_000640.2) spanning exon 1–2 and exons 6–7 and β-actin (internal PCR control) (Supplementary Table S3). Fold-difference in IL13Rα2 mRNA levels compared to 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 Luminesix 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/IL2Rγc−− (NSG) mice as approved by the COH Institute Animal Care and Use Committee. Intracranial 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-μm 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 ACS 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-μm slice interval and total volumes computed accordingly.

Results
Incidence of glioma-associated IL13Rα2 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 pas sing 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 IL13Rt2 on stem-like TS and serum-differentiated (DIF) glioma lines. For these studies, we compared IL13Rt2 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 IL13Rα2glio glioma lines U251T and U87 (19, 32), but not on the IL13Rα2glio T98 glioma and Daudi lymphoma cell lines (refs. 19, 32; Fig. 2A–C). It is important to note that IL13Rα2-specific commercial antibodies display significant differences in glioma IL13Rα2 detection. We find that clone B-D13 (Cell Sciences) and GW2245SA (GenWay) do not recognize an antigen on gliomas that is consistent with either IL13Rα2 mRNA expression or functional recognition of IL13Rα2 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-IL13Rt2 B-D13 antibody (Cell Sciences), to assess the frequency of IL13Rα2 expression on gliomas (18, 33). This inconsistency
may also underlie the lack of correlation between IL13Rα2 mRNA and protein expression for primary glioma biopsy specimens when the anti-IL13Rα2 B-D13 antibody (Cell Sciences) was used for IHC (34). We have therefore used the goat polyclonal anti-IL13Rα2 AF146 antibody (R&D Systems) for all experiments, as it is the only antibody tested that shows a consistent correlation between IL13Rα2 protein levels and mRNA expression [Western and flow cytometry vs. qRT-PCR; Fig. 2A–C, and Supplementary Fig. S1].

We next evaluated IL13Rα2 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 IL13Rα2 (>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). IL13Rα2 transcript levels, as measured by qRT-PCR, correlated with the observed IL13Rα2 protein expression (Fig. 2C). Our observations are thus in agreement with previous studies showing that the IL13Rα2 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 IL13Rα2 as assessed by flow cytometry and qRT-PCR (Fig. 2C and D). This observation suggested that the IL13Rα2 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 IL13Rα2 is expressed independently of glioma differentiation status. As exemplified by the IL13Rα2pos lines (PBT015-UPN033, PBT017-4, and PBT030), receptor expression is maintained on the stem-like TS (CD133pos Olig2pos), the 7- to 14-day serum-differentiated (GFAPpos; DIF), and the long-term serum-expanded cell populations (ADH; Fig. 2D and E). A small decrease in IL13Rα2 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, IL13Rα2neg primary lines (PBT003-4, PBT008, and PBT009-2) do not upregulate IL13Rα2neg upon differentiation and growth in serum-containing media (Fig. 2D and E). We concluded that although IL13Rα2 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 IL13Rα2, 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 IL13Rα2, as detected for the IL13Rα2neg tumors.

To assess the extent to which expression of IL13Rα2 on expanded primary cell lines correlates with the originating patient tumor, IL13Rα2 IHC was done on paraffin-embedded patient tumor tissue. As shown in Fig. 3, patient tumors

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 CD133pos 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.
with the highest overall IL13Rα2 expression levels (≥40% of cells positive) as detected by IHC (PBT015-UPN033, PBT017, and PBT030), generated both TS and DIF/ADH lines with correspondingly high IL13Rα2 expression (compare with Fig. 2D and E). Likewise, patient tumors with low IL13Rα2 levels (≤20% positive cells) gave rise to cell lines that lacked receptor expression (PBT008 and PBT009). Importantly, these findings indicated that expression levels of IL13Rα2 on expanded primary glioma cell lines are consistent with its expression by the originating patient tumor. However, on a cell-by-cell basis, IL13Rα2 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.

**IL13Rα2 expression renders GSCs susceptible to IL13-zetakine–mediated T-cell killing**

We next addressed whether IL13Rα2+ GSCs and differentiated tumor lines display comparable sensitivity to CAR-engineered CTL-mediated killing. For these studies, primary
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 IL13Rα2$^{++}$ 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% ± 9% with killing of TS ≥ 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 IL13Rα2$^{++}$ GSCs is not specific to a particular TS line, we showed in a similar set of experiments that IL13Rα2$^{++}$ 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 Th1 cytokines INF-γ and TNF-α (Fig. 4B, Supplementary Fig. S4). CTL coculture with PBT015-UPN033 TSs resulted in a significant increase in INF-γ and TNF-α production compared to control targets (Fig. 4B).

Figure 4. IL13-zetakine$^+$ CTL kill with comparable potency IL13Rα2$^{++}$ GSCs, differentiated cells, and established glioma cell lines. A, CRA measuring the lysis of IL13Rα2$^{++}$ 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 IL13Rα2$^{++}$ CD8$^+$ CD19$^-$ 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 clone 3C12 as in (B) upon preincubation of the CTL with increasing concentrations of blocking rat anti-human IL13 (left) or PBT015-UPN033 TS target cells with rhIL13 (right). D, CRA measuring lysis of IL13Rα2$^{++}$ glioma lines PBT003-4 TS and T98 cells versus IL13Rα2$^{++}$ PBT015 by the indicated CD8$^+$ IL13-zetakine$^+$ CTL at increasing E:T ratios. Mean ± SD values of wells (n = 6) are depicted.
slightly less Tc1 cytokine production as compared with serum-differentiated (DIF) cells (average ± SD decrease in INF-γ for TS vs. ADH and DIF is 42% ± 7% and 14% ± 2%, respectively; average decrease in TNF-α for TS vs. ADH and DIF is 58% ± 2% and 45% ± 13%, respectively). This decrease in T-cell–mediated INF-γ and TNF-α cytokine release is observed on engagement of all IL13Ro2<sup>pos</sup> primary glioma lines tested (average 2- to 3-fold decrease) (Supplementary Fig. S4).

Several lines of evidence confirmed that the IL13-zetakine<sup>-</sup> CTL killing and Tc1 cytokine responses we observed are specific to engagement of IL13Ro2<sup>-</sup> tumor antigen. First, IL13-zetakine<sup>-</sup> CTL clones were not activated for killing or cytokine production when coincubated with the IL13Ro2<sup>pos</sup> PBT003-4 TS and T98 glioma lines or the IL13Ro2<sup>pos</sup> LCL line (Fig. 4A, B, and D, and data not shown). Second, blocking CAR-IL13Ro2<sup>-</sup> engagement by either preincubation of PBT015-UPN033 TS targets with recombinant human IL13 (rhIL13), or IL13-zetakine<sup>-</sup> 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 IL13Ro2 tumor–associated antigen is necessary for IL13-zetakine<sup>-</sup> CTL killing (19). Importantly our data establishes that both IL13Ro2<sup>pos</sup> 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<sup>-</sup> CTL can ablate the tumor-initiating activity of IL13Ro2<sup>pos</sup> 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 IL13Ro2<sup>pos</sup> 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<sup>-</sup> 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<sup>-</sup> CTL, but not PBS or control CD19<sup>-</sup> CTL, ablated the engraftment potential of PBT017-4 cells (Fig. 5A). Similarly, IL13-zetakine<sup>-</sup> CTL, but not PBS or control CD19<sup>-</sup> 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<sup>-</sup> CTL do not ablate the tumor-initiating activity of the IL13Ro2<sup>pos</sup> line, PBT003-4 TSs (Fig. 5C), consistent with the lack of in vitro killing of PBT003-4 when cocultured with IL13-zetakine<sup>-</sup> CTL (Fig. 4D). This study showed that IL13-zetakine<sup>-</sup> CTL can eliminate the tumor-initiating activity of both IL13Ro2<sup>pos</sup> GSCs and differentiated subpopulations.

**Regression of established GSC TS-initiated xenografts after adoptive transfer of IL13-zetakine<sup>-</sup> T cells**

We next evaluated the capacity of IL13-zetakine<sup>-</sup> CTL to induce regression of established GSC TS-initiated tumors. For these studies, we examined the antitumor efficacy of IL13-zetakine<sup>-</sup> T cells against IL13Ro2<sup>pos</sup> 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 (FlLUC; EGFP-flLUC; ref. 9). EGFP-flLuc<sup>+</sup> PBT030-2 or nonengineered 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<sup>-</sup> CD8<sup>+</sup> CTL clone 2D7 or control CD19<sup>-</sup> CD8<sup>+</sup> CTL clone E8. By comparing FlLuc<sup>+</sup> 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<sup>-</sup> CTL, as compared with control CD19<sup>-</sup> CTL, mediate statistically significant anti-glioma activity against both PBT030-2- and PBT017-4–established tumors.

The ability of IL13-zetakine<sup>-</sup> CTL to regress GSC-initiated glioblastoma tumors is consistent with our previous observations that IL13-zetakine<sup>-</sup> CTL can kill IL13Ro2<sup>pos</sup> 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<sup>-</sup> CTL, we sought to understand the etiology of tumor escape by evaluating the expression of IL13Ro2 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<sup>-</sup> CTL– and control CD19<sup>-</sup> CTL–treated tumors express comparable levels of IL13Ro2 (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<sup>+</sup> 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<sup>-</sup> CTL–mediated tumor targeting. Although the largest IL13-zetakine<sup>-</sup> 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
IL13-zetakine⁺ CTL has the capacity to reduce tumor growth and tumor burden of established IL13Rα2 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 IL13Rα2 expressing tumor cells.

Discussion

Although immunotherapy strategies targeting IL13Rα2 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 IL13Rα2-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 IL13Rα2 expression is not limited to differentiated glioma cells and that IL13Rα2 targeting can potentially eliminate the refractory GSC component of malignant gliomas. We show that GSC TSs derived from IL13Rα2⁺⁺⁺ glioma tumors express IL13Rα2 and that its expression renders the IL13Rα2⁺ 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 IL13Rα2⁺⁺⁺ GSCs and, further, mediate robust antitumor activity and limit progression of established IL13Rα2⁺⁺⁺ 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.
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 IL13Rα2-directed therapies is not due to the absence of IL13Rα2 expression by all GSC subpopulations, but rather that other mechanisms of escape may also be at play. Furthermore, we show that IL-13-zetakine-2 CTLs can target and kill IL13Rα2pos GSCs and regress established GSC-initiated tumors. However, for established tumors, IL13Rα2 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 IL13Rα2pos 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 IL13Rα2 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 IL13Rα2 on tumor-infiltrated macrophages correspondingly inhibits TGF-β production and restores the antitumor activity of CD8+ T cells. In these ways, targeting IL13Rα2 expressed by other cells in the tumor microenvironment may also enhance the efficacy of clinically targeting this receptor.

Importantly, the heterogeneity of IL13Rα2 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 IL13Rα2 is expressed by a subset of glioma stem-like–initiating populations susceptible to cell immunotherapy highlight the relevance of translational strategies targeting IL13Rα2 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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Importantly, the heterogeneity of IL13Rα2 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 IL13Rα2 is expressed by a subset of glioma stem-like–initiating populations susceptible to cell immunotherapy highlight the relevance of translational strategies targeting IL13Rα2 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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Christine E. Brown, Renate Starr, Brenda Aguilar, et al.


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