Recognition and Killing of Brain Tumor Stem-Like Initiating Cells by CD8+ Cytolytic T Cells

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Abstract

Solid tumors contain a subset of stem-like cells that are resistant to the cytotoxic effects of chemotherapy/radiotherapy, but their susceptibility to cytolytic T lymphocyte (CTL) effector mechanisms has not been well characterized. Using a panel of early-passage human brain tumor stem/initiating cell (BTSC) lines derived from high-grade gliomas, we show that BTSCs are subject to immunologic recognition and elimination by CD8+ CTLs. Compared with serum-differentiated CD133low tumor cells and established glioma cell lines, BTSCs are equivalent with respect to expression levels of HLA class I and ICAM-1, similar in their ability to trigger degranulation and cytokine synthesis by antigen-specific CTLs, and equally susceptible to perforin-dependent CTL-mediated cytolysis. BTSCs are also competent in the processing and presentation of antigens as evidenced by the killing of these cells by CTL when antigen is endogenously expressed. Moreover, we show that CTLs can eliminate all BTSCs with tumor-initiating activity in an antigen-specific manner in vivo. Current models predict that curative therapies for many cancers will require the elimination of the stem/initiating population, and these studies lay the foundation for developing immunotherapeutic approaches to eradicate this tumor population. [Cancer Res 2009;69(23):OF1–8]

Introduction

Many tumors initially respond to conventional therapies such as radiation and chemotherapy, only to recur as a more therapeutically resistant malignancy. Recurrence has been attributed to the preferential survival of a subpopulation of tumor cells, termed cancer stem cells (CSC), which have the capacity for self-renewal, multilineage differentiation, and tumor initiation (1). Tumor cell populations enriched for CSCs have been found to be resistant to ionizing radiation due to alterations in cell signaling pathways involved in DNA damage tolerance and repair (2, 3) and resistant to chemotherapy due to increased expression of drug efflux pumps and decreased activity of apoptotic pathways (2, 4). Thus, the curative potential of anticancer therapies hinges, in part, on the development of therapeutic modalities that can eradicate this formidable subset of stem cell–like malignant cells.

We hypothesize that cellular immunotherapy might effectively target the CSC population because cytolytic T lymphocyte (CTL)-mediated killing is independent of target cell proliferation status and, thus, theoretically equally potent at eliminating both the relatively quiescent tumor stem population as well as the more actively dividing differentiated tumor cell. In support of this, CD8+ CTL specific for minor histocompatibility antigens can prevent engraftment of human acute myeloid leukemia stem cells in immunodeficient mice (5). However, our knowledge about the susceptibility of stem cell–like malignant cells within solid tumors to CTL effector mechanisms is rudimentary.

CSCs have been prospectively identified in solid tumors, including brain, breast, colon, and pancreatic tumors, based on differential marker expression (6). Brain tumor stem/initiating cells (BTSCs) in particular can be enriched from many high-grade gliomas based on expression of the cell surface marker CD133, and in these tumors, it is the CD133+ population that has the most potent tumor-initiating activity (3, 7). Importantly, BTSCs that expanded in serum-free neural stem cell medium maintain their stem-like characteristics, preserve gene expression profiles that closely mirror the primary tumor, and initiate de novo tumors in immunodeficient mice that are phenocopies of the originating tumor (7–10). Here, we assessed the susceptibility of CSCs that were expanded from a panel of primary high-grade human gliomas to CTL-mediated effector mechanisms and compared their sensitivity to differentiated and established glioma cell lines.

Materials and Methods

Cell lines. Human cytomegalovirus (CMV) pp65-specific CTL lines were generated from peripheral blood mononuclear cells (PBMC) of consented healthy CMV-seropositive donors participating in Internal Review Board–approved protocols. The HLA-A2–restricted pp65-specific CTL bulk line was derived from the fluorescence-activated cell sorted CD62L+CD45RO+ T-cell fraction stimulated with autologous irradiated PBMC transiently expressing pp65 (4:1) and 5 units/mL of recombinant human interleukin-2 (Chiron) once a week for 3 wk. The HLA-A24–restricted pp65-specific T-cell clone was generated as described previously (11). T cells were further expanded and maintained as previously described (12).

Glioma specimens, graded according to WHO-established guidelines (Supplementary Table S1), were obtained from patients in accordance with Institutional Review Board–approved protocols. Mined tumor specimens were implanted s.c. in the flank of nonobese diabetic/severe combined immunodeficient (NOD-scid) mice, and the remaining tumor was dissociated into single cells using 400 units/mL of collagenase III (Sigma-Aldrich) in neural stem cell medium [DMEM/F12 (Irvine Scientific), 1:50 B27 (Invitrogen),
were analyzed by flow cytometry using anti-CD133 (12). Changes in expression under serum growth conditions were normalized to actin. Blots detecting CD133, Olig2, GFAP, and β-III tubulin in TS and DIF cells. Fold quantitation (ng/mL) of VEGF secreted by TS and DIF cells. Cytotoxicity and cytokine assays.

Four-hour chromium release assays (CRA) and luciferase-based cytotoxicity assays (LCA) were performed as previously described (15, 16). When specified, tumor cells were peptide loaded in neural stem cell medium at a final concentration of 10 μg/mL peptide for 2 h at 37°C. For concanamycin A (CMA) inhibition, CTLs were loaded in neural stem cell medium at a final concentration of 10 μg/mL. Cytotoxicity and cytokine assays were performed as described (17). For the four-hour chromium release assay, tumor cells were stained with 0.1 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE) as per the manufacturer’s instructions. Blots were imaged on the Odyssey Infrared Imaging System (LI-COR) and band intensities were quantified using Odyssey v2.0 software (LI-COR).

Cytotoxicity and cytokine assays. Four-hour chromium release assays (CRA) and luciferase-based cytotoxicity assays (LCA) were performed as previously described (15, 16). When specified, tumor cells were peptide loaded in neural stem cell medium at a final concentration of 10 μg/mL peptide for 2 h at 37°C. For concanamycin A (CMA) inhibition, CTLs were resuspended at 10⁶ cells/mL and incubated for 2 h at 37°C in the presence or absence of CMA (Calbiochem EMD Chemicals, Inc.) before coculture with tumor cells at a 10:1 E:T ratio. CMA (Calbiochem EMD Chemicals, Inc.) before coculture with tumor cells at a 10:1 E:T ratio.

Cytokine production was measured by culturing T cells with tumor at a 10:1 E:T ratio. After 20 to 24 h of incubation, supernatants were harvested and assayed using Luminex multiplex bead technology (Upstate), and output data were analyzed via the Bio-Plex Manager 4.0 software (Bio-Rad).

Tumor xenografts and in vivo biophotonic imaging. Mice were maintained under specific pathogen-free conditions, and all procedures were performed with 6- to 8-week-old NOD-scid mice as approved by the City of Hope Institute Animal Care and Use Committee. Intracranial (i.c.) tumor xenografts and biophotonic imaging were performed as previously described (12).

DNA constructs. The CMV-pEGFP-plantluc_pHIV7 lentiviral construct encodes an engineered fusion between enhanced green florescent protein (EGFP) and firefly luciferase (fluc) separated by a three-glycine linker (EGFP-fluc) expressed under the control of the CMV-1 enhancer/promoter. The EF1α promoter and CMV p65 (gift from Dr. John Zaia, City of Hope National Medical Center) separated by the 2A self-cleaving peptide (14) expressed under the control of the human elongation factor 1α promoter. The pHIV7 vector backbone was a gift of J.K. Yee (City of Hope National Medical Center). Construct sequences are provided on request.

Flow cytometric analysis. Cell surface phenotypes were assayed as previously described (15) using either FITC-conjugated anti-CD31, anti-CD45, anti-CD54, anti-HLA-DR, or anti-HLA-ABC (BD Biosciences) or phycoerythrin-conjugated mouse anti-human CD133/2 and anti-human CD133/2 (Miltenyi Biotec). Percentage of immunoreactive cells was calculated using the subtraction method via FCS Express version 3 software (De Novo Software).

Protein analysis. Western blots were probed with rabbit polyclonal anti-actin (Rockland); goat polyclonal anti-Olig2 (R&D Systems); and mouse monoclonal anti-β-III tubulin (Millipore), anti-CD133 (Miltenyi Biotec), and anti-glial fibrillary acidic protein (GFAP; Sigma-Aldrich) antibodies as per the manufacturers’ instructions. Blots were imaged on the Odyssey Infrared Imaging System (LI-COR) and band intensities were quantified using Odyssey v2.0 software (LI-COR).

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5 μg/mL heparin (Abraxis Pharmaceutical Products), and 2 mmol/L t-glutamine (Irvine Scientific); refs. 12, 13. Tumor spheres (TSs) were expanded from either dissociated s.c. xenografts or primary tumor cells in neural stem cell medium supplemented with 20 ng/mL epidermal growth factor (EGF; R&D Systems), 20 ng/mL basic fibroblast growth factor (R&D Systems), and 20 ng/mL leukemia inhibitory factor (Millipore) replenished in the culture medium twice a week. TSs were dissociated with accutase (Innovative Cell Technologies) and differentiated in DMEM:F12, 2 mmol/L t-glutamine, 25 mmol/L HEPES, and 7% FCS for 7 to 14 d. U251 glioblastoma adherent cells (gift from Dr. Waldemar Debinski, Pennsylvania State University) were grown in DMEM (Irvine Scientific) supplemented with 10% FCS, 2 mmol/L t-glutamine, and 25 mmol/L HEPES.

DNA constructs. The CMV-pEGFP-plantluc_pHIV7 lentiviral construct encodes an engineered fusion between enhanced green florescent protein (EGFP) and firefly luciferase (fluc) separated by a three-glycine linker (EGFP-fluc) expressed under the control of the CMV-1 enhancer/promoter. The EF1α promoter and CMV p65 (gift from Dr. John Zaia, City of Hope National Medical Center) separated by the 2A self-cleaving peptide (14) expressed under the control of the human elongation factor 1α promoter. The pHIV7 vector backbone was a gift of J.K. Yee (City of Hope National Medical Center). Construct sequences are provided on request.

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Immunohistochemistry. Brains were harvested, fixed, and embedded in paraffin as described previously (12). Horizontal brain sections (10 μm) were deparaffinized, underwent citrate antigen retrieval, and stained with either human-specific anti-B23 antibody or CMV pp65-specific antibody (Leica Microsystems) followed by detection using the EnVision kit (Vector Laboratories, Inc.) with 50% hematoxylin counterstain.

Results

TSs expanded from glioma explants exhibit stem cell–like characteristics. We used two independent methods to expand short-term TS cultures of BTSCs from primary high-grade gliomas. The first approach involved the dissociation of tumor-derived tissue and direct culturing in serum-free neural stem cell medium. TS cultures derived by this approach include those from PBT008 (patient brain tumor 008) and PBT009. The second approach involved s.c. implantation of minced tumor specimens in the flank of NOD-scid mice to enrich in vivo for the tumor-initiating population (18). The s.c. tumors were subsequently excised and dissociated and the cells were then expanded in neural stem cell medium. TS cultures derived by this approach include those from PBT003, PBT009, and PBT017, all of which were passaged two to four times s.c. (scp2–4) in the flank of mice before they were dissociated and expanded in vitro.

The expanded TS exhibited cytogenetic abnormalities commonly observed in human glioma malignancies (Supplementary Table S2), confirming that the cells were indeed human tumor derived. The rate of secondary TS formation, an assessment of in vitro self-renewal potential, was evaluated by limiting dilution and ranged from 3% to 11% (Supplementary Table S3). PBT003, PBT008, and PBT009 TSs primarily grew as spheroids with few attached cells and displayed consistently high CD133 expression (Fig. 1A). Although PBT017 scp4 grew as unattached spheres and expressed CD133 for the first few in vitro passages (Fig. 1A), following subsequent expansion (more than six passages) this line grew primarily as an adherent monolayer with a concomitant downregulation of CD133 expression (data not shown). Distinct subtypes of glioma TS lines that differ in spherical growth and CD133 expression but maintain stem cell marker expression and tumor-initiating activity have also been reported by others (8, 9).

Expanded TSs exhibit stem cell–like properties as judged by expression of established stem cell markers. TS cells express significant, although varying, levels of CD133 (Fig. 1A), β-catenin, BMI1, nestin, SOX2 (Supplementary Fig. S1), and Olig2 (Fig. 1B). In contrast, when the TSs were transferred to serum-containing medium, they grew as an adherent monolayer and underwent differentiation as evidenced by a reduction in CD133 and Olig2 expression (Fig. 1A and B). Moreover, upregulation of the astrocytic marker GFAP and the neuronal marker β-III tubulin (Fig. 1B) was observed for all TS lines, except serum-differentiated PBT009. Heterogeneity in serum-induced differentiation marker expression, particularly GFAP, has been reported previously and reflects differences between distinct subgroups of glioma-derived BTSC lines (9). In concordance with previous reports, we also find that the TSs secrete 10- to 30-fold higher levels of vascular endothelial growth factor (VEGF) compared with differentiated cultures (Fig. 1C; ref. 19).

To evaluate their in vivo tumorigenicity, TSs were implanted i.c. in NOD-scid mice. Expanded TS lines (PBT003 scp4, PBT009, PBT009 scp4, and PBT017 scp4) were found to initiate infiltrative tumors exhibiting pathologic features of clinical gliomas at cell doses as low as 10^2 to 10^3 (Fig. 1D; Supplementary Table S3; Supplementary Fig. S2A and B). As exemplified by PBT003 TS,
orthotopic tumors displayed similar histologic features to the primary tumor with many common diagnostic features of glioblastoma (Fig. 1D). Cells derived from PBT008, however, showed reduced tumorigenicity compared with the other expanded TS cultures, with mice remaining asymptomatic at 5 to 7 months and only a modest number of diffuse tumor cells being detected at this end point by immunohistochemistry (data not shown).

Such differences in tumor-initiating potential and engraftment characteristics between tumor specimens have been reported by others (9). In summary, the low-passage TS lines characterized in this study exhibit glioma cancer stem/initiating cell phenotypes and the ability to initiate infiltrative orthotopic tumors.

**Cell surface molecules required for T-cell/tumor immunologic synapse formation are expressed by BTSCs.** It is well documented that tumors can escape T-cell–mediated elimination by downregulating molecules essential for immune recognition (20, 21). Our ability to expand sufficient numbers of low-passage TS and matched differentiated cells enabled us to carefully examine these distinct cell populations for potential differences in molecules that are essential for T cell/target synapse formation. Although there are mixed reports regarding the basal level of MHC expression on low-passage glioma stem cell lines (22, 23), we find that CD133+ TSs and CD133− serum-differentiated lines express significant and comparable levels of MHC I and ICAM-1/CD54, suggesting that expression of these molecules is retained in the BTSC subset (Fig. 2A). By comparison, MHC II (HLA-DR) was expressed by only PBT009 and PBT017 TSs, with expression levels decreasing following serum-induced differentiation (Fig. 2A). Gliomas frequently express cell surface MHC II (24), and our data suggest that, for these gliomas, MHC II is also expressed by the BTSC population.

![Figure 3](image3.jpg) **Figure 3.** CMV-specific CTLs kill pp65 peptide-loaded PBT008 tumor stem cells equivalent to differentiated and established glioma cell lines. LCA (A) and CRA (B) measuring the lysis of CMV pp65 peptide (NLVPMVATV) versus control influenza M1 peptide (GILGFVFTL)–loaded PBT008-GL+ (EGFP-fluc) TS, serum-differentiated PBT008-GL− (DIF), or U251-GL+ cells by CD8+ CMV pp65-specific CTL at increasing E:T ratios. Points, mean (n = 6 wells); bars, SD. *, P < 0.006, when comparing M1 versus pp65 peptide-loaded targets using unpaired Student’s t test. C, IFN-γ and TNF-α produced by CTL after coculture with specified peptide-loaded tumor targets. D, flow cytometry–based killing assay monitoring the loss of viable CD133low, CD133high, or total PBT008 TS cells at increasing E:T ratios. Inset, PBT008 TS CD133 expression and percentage CD133high (48%) and CD133low (51%) cells.

![Figure 4](image4.jpg) **Figure 4.** Perforin-dependent killing by CMV-specific CTL is equivalent for pp65 peptide-loaded PBT003 TS and DIF cells. A, lysis of CMV pp65 peptide (QYDPVAALF)–loaded PBT003-GL+ (EGFP-fluc) TS or DIF cells by the CD8+ pp65-specific CTL at increasing E:T ratios was determined by LCA. Points, mean (n = 6 wells); bars, SD. *, P = 0.0004, when comparing ± pp65 peptide using unpaired Student’s t test. B, IFN-γ and TNF-α produced by CTL after coculture with specified tumor targets. C, flow cytometric detection of CD107a on CD8+ CTL after coculture with tumor cells as in A at a 2:1 E:T ratio in the presence (bottom) and absence (top) of CMV peptide. D, inhibition of LCA-measured killing of pp65-loaded PBT003-GL+ scp4 TS and DIF tumor targets at a 10:1 E:T ratio on preincubation of CD8+ pp65-specific CTL with increasing concentrations of CMA. At all CMA concentrations, P < 0.003, when compared with no CMA using unpaired Student’s t test.
We sought to verify our findings on noncultured primary glioma specimens and, similar to Anderson and colleagues (24), found that all high-grade glioma specimens expressed detectable but heterogeneous levels of MHC and ICAM-1, often with distinct subsets of cells displaying very low cell surface expression (Fig. 2B). Expression of these cell surface molecules cannot be attributed to contaminating hematopoietic cells because freshly dispersed primary tumor samples did not contain significant numbers of CD45+ cells (Supplementary Fig. S3). To specifically evaluate MHC and ICAM-1 levels on the putative BTSC population, we evaluated tumor samples with anti-CD133. Our analysis focused on PBT009, PBT024, and PBT025, as these samples had the greatest CD133 expression (Fig. 2B). Surprisingly, the CD133+ cells exhibited the highest expression of all three cell surface molecules involved in T-cell/tumor cell immunologic synapse formation (Fig. 2C). Staining for CD31 confirmed that the CD133+ cells were tumor derived and not endothelial progenitors. Although discrepancies in the frequency of MHC expression for noncultured high-grade glioma specimens have been reported (24, 25), our results show that the CD133+ BTSC population is not deficient in MHC I/II and ICAM-1 expression when compared with the CD133− bulk tumor population and that there is instead a positive correlation between CD133 and molecules important for immune recognition.

**MHC I–restricted CD8+ CTL recognition and lysis of CMV peptide-pulsed CD133+ and CD133− tumor populations.** Tumor-related factors, such as resistance to perforin and/or expression of inhibitory cell surface molecules, and secretion of immunosuppressive cytokines, may also affect the susceptibility of tumors to CTL-mediated killing (26). To determine whether the BTSC population is susceptible to CTL killing mechanisms, we used HLA-A2+ PBT008 and U251T (tumorigenic subclone of glioma line U251) as target cells and a primary human HLA-A2–restricted CMV pp65–specific CD8+ T-cell clone (TCR+, CD3+, perforin+, granzyme), which recognizes the HLA-A2–bound pp65 immunodominant peptide NLVPMVATV (Supplementary Fig. S4). Two cytolytic assays were used in these experiments: (a) a LCA (16) that reads out cell death based on decreases in cellular ATP and O2 levels and (b) a CRA that reads out perforin-mediated plasma membrane disruption. Using each assay, CMV pp65–specific CTLs killed with comparable potency the pp65 peptide-loaded PBT008 TS, matched serum-differentiated tumor (PBT008 DIF), and U251T lines (average difference in cytotoxicity at all E:T ratios was 12.2 ± 5.7% with killing of TS ≥ DIF lines; Fig. 3A and B). T-cell activation–dependent cytokine production of IFN-γ and tumor necrosis factor-α (TNF-α) was also shown to be similar upon engagement of either BTSC or differentiated targets (Fig. 3C), although expression levels of costimulatory molecules such as NKG2D and CD28 ligands differed between U251T and the PBT008 lines (Supplementary Fig. S5). Because CD133 expression levels have been found to correlate with self-renewal potential and tumor initiation (3, 7, 8), we also used a flow cytometry–based killing assay to specifically compare CTL-mediated elimination of the CD133high versus CD133low cells within the TS population. We found that both TS populations were killed equivalently by CD8+ CTL (Fig. 3D).

We extended these observations to the HLA-A24+ PBT003 scp4 TS and differentiated lines (Supplementary Fig. S6A). Here, too, we observed that an HLA-A24–restricted CMV pp65–specific T-cell clone (TCR+, CD3+, perforin+, granzyme) killed with comparable efficiency both the BTSC and differentiated targets loaded with the pp65...
HLA-A24 binding peptide QYDPVAALF (average difference in cytotoxicity at all E:T ratios was 4.6 ± 1.5% with killing of TS ≥ DIF lines; Fig. 4A). Production of IFN-γ and TNF-α by the CTL clone was also similar when the T cells were cocultured with either PBT003 TS or serum-differentiated tumor cells (Fig. 4B) and occurred in the absence of tumor cell surface expression of either CD28 or NKG2D costimulatory ligands (Supplementary Fig. S5).

The primary mechanism by which CD8+ T cells mediate cytolytic activity against target cells is via the intrinsic granule exocytosis pathway involving the directed release of preformed lytic vesicles containing perforin and granzymes (27). Cell surface expression of the lysosomal-associated membrane proteins CD107a and CD107b, which can be detected by flow cytometry, is associated with antigen-dependent perforin/granzyme granule exocytosis (17). When challenged with either TS or serum-differentiated PBT003 (scp4) TSs (2 × 10^5) expressing either EGFP:fluc (TS-GL) or EGFP:fluc and the CMV pp65 antigen (TS-GL-pp65) were coinjected i.c. with 2 × 10^6 pp65-specific CTL or PBS into NOD-scid mice (n = 6 mice per group). A, flow cytometric analysis of TS-GL and TS-GL-pp65 with only 57.2% of the cells expressing the transgenes (+/−) or TS-GL-pp65 with 91.9% of the cells expressing the transgenes (++). B, points, mean biophotonic flux of the i.c. tumors over time was determined by Xenogen imaging; bars, SE. *, P = 0.017, when comparing TS-GL-pp65++ tumors that had been coinjected with CTL versus PBS at day 47 using an unpaired Student's t test; **, P = 0.036, when comparing TS-GL-pp65++ tumors that had been coinjected with CTL versus PBS at day 48 using an unpaired Student's t test. C, representative biophotonic images of mice from each group at day 47. D, tiled images of horizontal brain sections from representative mice that had received TS-GL-pp65 cells with PBS (top) or CTL (bottom) and were stained with H&E (left) or for the B23 human cell marker (middle two) or the CMV pp65 antigen (right) by immunohistochemistry. Right, anti-B23 and anti-pp65 immunohistochemistry near the injection site.

Figure 6. CMV-specific CTLs ablate the tumor initiation potential of pp65-expressing TS. PBT003 (scp4) TSs (2 × 10^5) expressing either EGFP:fluc (TS-GL) or EGFP:fluc and the CMV pp65 antigen (TS-GL-pp65) were coinjected i.c. with 2 × 10^6 pp65-specific CTL or PBS into NOD-scid mice (n = 6 mice per group). A, flow cytometric analysis of TS-GL and TS-GL-pp65 with only 57.2% of the cells expressing the transgenes (+/−) or TS-GL-pp65 with 91.9% of the cells expressing the transgenes (++). B, points, mean biophotonic flux of the i.c. tumors over time was determined by Xenogen imaging; bars, SE. *, P = 0.017, when comparing TS-GL-pp65++ tumors that had been coinjected with CTL versus PBS at day 47 using an unpaired Student's t test; **, P = 0.036, when comparing TS-GL-pp65++ tumors that had been coinjected with CTL versus PBS at day 48 using an unpaired Student's t test. C, representative biophotonic images of mice from each group at day 47. D, tiled images of horizontal brain sections from representative mice that had received TS-GL-pp65 cells with PBS (top) or CTL (bottom) and were stained with H&E (left) or for the B23 human cell marker (middle two) or the CMV pp65 antigen (right) by immunohistochemistry. Right, anti-B23 and anti-pp65 immunohistochemistry near the injection site.
tumor cells that had been loaded with pp65 peptide, CD8+ T cells degranulate equally as assessed by cell surface mobilization of CD107a (Fig. 4C). CMA, an inhibitor of vacuolar-type H+ ATPase, is a highly specific inhibitor of perforin-dependent cytolysis that promotes the degradation of perforin (28). The T-cell lytic activity against both the TS and differentiated targets was strongly inhibited with CMA concentrations of ≥5 nmol/L (Fig. 4D). We therefore conclude that glioma CD133+ TS and CD133- differentiated tumor populations are equally sensitive to CTL-mediated cytolyis involving granule exocytosis and perforin.

Glioma CD133+ TSs and CD133- tumor populations can process and present antigen for CD8+ CTL recognition. Experiments that examine the sensitivity of peptide-loaded tumor cell subpopulations to CTL recognition do not test the integrity of the intracellular pathways required to display antigenic peptides. Thus, to assess whether the protein processing machinery is sufficiently intact for the BTSC population to process and present antigen for CD8+ CTL recognition, we engineered U251T, PBT003, and PBT008 glioma targets to endogenously express the CMV pp65 antigen. For these studies, a bicistronic lentiviral construct was made encoding CMV pp65 and a fusion of the EGFP and ffLuc reporter proteins, separated by the 2A self-cleaving peptide (Fig. 5A; ref. 14). Lentiviral transduction of cells results in the expression of both protein products (pp65 and EGFP:ffLuc) and ensures that all EGFP:ffLuc+ cells express the pp65 antigen. CMV-specific CTLs were found to kill with similar potency pp65-expressing TS, matched serum-differentiated tumor, and the U251T glioma line (average difference in cytotoxicity at all E:T ratios was 12.3 ± 8.2%; Fig. 5B). These data suggest that antigen processing and presentation by BTSC are sufficiently intact for recognition and killing by CD8+ CTL.

**CTL can ablate the tumor-initiating potential of BTSCs in vivo.** To test whether CTL can eliminate all tumor-initiating activity of the BTSCs, pp65+ EGFP:ffLuc+ PBT003 TSs and pp65-specific CTLs were coinjected i.c. into NOD-scid mice. We hypothesized that a rare tumor-initiating population (≥0.5%) that is resistant to CTL-mediated cytolysis would be detected by this in vivo implantation assay because PBT003 is highly tumorigenic at doses as low as 1,000 cells (Supplementary Table S3), and 2 × 10^6 PBT003 cells were injected i.c. for these assays. As shown in Fig. 6, coinjection of CMV-specific CTL with pp65-expressing PBT003 scp4 TSs ablated the tumor initiation activity of antigen-positive PBT003 BTSCs (i.e., all EGFP:ffLuc+ pp65+ cells). The specificity of this system was shown using PBT003 scp4 TS cells that were only 57% positive for pp65/EGFP:ffLuc (Fig. 6A). Although all pp65+ antigen-positive (hence EGFP:ffLuc+) tumor cells were ablated, pp65+ tumor cells were resistant to the pp65-specific CTL and efficiently engulfed (Fig. 6B-D). This result establishes that direct recognition of antigen-expressing tumor cells by CTLs is required to ablate tumor initiation and negates the possibility of bystander effector mechanisms. Because tumor initiation is a hallmark feature of the CSC population, the inability of antigen-expressing PBT003 TS to initiate tumors when coinjected with antigen-specific CTL shows that T cells are capable of efficiently eradicating the tumor-initiating population of malignant glioma.

**Discussion**

The CSC hypothesis has profound implications for predicting the curative potential of emerging novel therapeutic modalities. CSCs have been shown to differ from the differentiated bulk tumor population in their genetic profile, cytokine production, and cellular interactions within the tumor (10, 19, 29). The clinical significance of these differences resides in the resistance of the CSC population to conventional therapies, including radiation and chemotherapy. Here, we find no discernable difference in the capacity of brain tumor CSCs and the differentiated glioma tumor population to be targeted and killed by T-cell-mediated effector mechanisms. Our studies establish that BTSCs are highly sensitive to CTL-mediated perforin-dependent killing mechanisms and are consistent with reports that the BTSC population is also sensitive to natural killer-mediated lysis (22, 23). We find evidence of equal expression of surface markers involved in immunologic synapse formation, such as MHC I and the cell adhesion molecule ICAM-1. Likewise, IFN-γ-mediated upregulation of MHC I on gliomas has also been shown to be comparable for CD133+ and CD133- populations (23). We further show that BTSCs can process and present cytosolic protein antigens on MHC I for recognition by CD8+ CTL. Moreover, we show that CD8+ CTL can eliminate all cellular populations capable of tumor engraftment.

In this study, we have used CMV pp65-specific CD8+ T cells and MHC I-matched tumor lines to evaluate susceptibility to cytolytic killing of peptide-loaded or pp65-expressing BTSCs. This investigational platform was used as it minimized the contribution of differences in tumor-associated antigen expression by patient-derived glioma specimens and variation of TCR affinities for tumor antigens that are also self-antigens. Moreover, the recent detection of CMV viral antigens in a high percentage of gliomas (30), and the development of immune-based strategies aimed at targeting reactivated CMV antigens in gliomas (31), highlights the relevance of this model platform as well as our findings that BTSC can process and present CMV antigens for CTL recognition.

The definition and prospective identification of CSCs continue to evolve, and for this reason, our study did not rely on a single-cell source or marker to define this population; that is, based on TS growth, CD133 expression, and tumor-initiating activity, we found no deficiency in the targeting of these cells by CTL. TSs that were expanded in serum-free medium in the presence of EGF and FGF display stem–cell–like characteristics and retain molecular expression profiles that more closely resemble the primary tumor than do tumor cells grown in standard serum-containing medium (10). Indeed, substantial evidence suggests that serum-derived tumor lines are not optimal for translating effective therapies to the clinic (32). Thus, our demonstration that T cells can efficiently target and kill in vitro–expanded TSs represents an important step in the development of brain tumor models that more closely mimic the pathology and therapeutic response of the primary tumor.

Because glioma-derived TSs are highly invasive in orthotopic models of tumor engraftment (9, 33) and interact closely with endothelial cells in the perivascular niche (29), it remains critical to assess the ability of CTL to home to disparate sites of infiltrating tumor. In general, CTLs are capable of surveying the central nervous system (CNS) parenchyma (34), and progress has been made toward exploiting CTL surveillance in a therapeutic context. Our group has shown that i.v. administered CTLs traffic to and infiltrate sites of glioma tumor burden within the CNS due in part to the secretion of CCL2/MCP-1 by these tumors (35). Furthermore, we have observed that glioma-specific CTL clones...
administered into a tumor resection cavity of patients can migrate to sites of disease in the contralateral hemisphere (36).

The prognosis of patients with high-grade brain tumors remains grim, and current thinking toward the development of curative therapy is likely to require eradication of the BTSC population. It is well established that gliomas have evolved many mechanisms to evade the immune system, and these hurdles remain for immune-based therapies. However, our studies establish that the CSC target is intrinsically susceptible to immune-based killing and support the premise that efforts to overcome these other obstacles associated with immune-targeting of brain tumors are well spent.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Recognition and Killing of Brain Tumor Stem-Like Initiating Cells by CD8+ Cytolytic T Cells

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