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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 chemo/radio-therapy, 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 cell (BTSC) lines derived from high-grade gliomas, we demonstrate that BTSCs are subject to immunologic recognition and elimination by $CD8^+$ CTLs. Compared to serum differentiated $CD133^{low}$ 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.

Keywords

cancer stem cell; brain tumor stem/initiating cell; tumor sphere; CD133; $CD8^+$ cytolytic T lymphocyte

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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 (CSCs) that have the capacity for self-renewal, multi-lineage 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 anti-cancer therapies hinges, in part, upon 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, since 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 regarding 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 (BTSC) 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, BTSC 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 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 CMV pp65-specific CTL lines were generated from peripheral blood mononuclear cells (PBMC) of consented healthy CMV-seropositive donors participating on Internal Review Board-approved protocols. The HLA-A2 restricted pp65-specific CTL bulk line was derived from the fluorescence-activated cell sorted (FACS) $CD62L^-CD45RO^+$ T cell fraction stimulated with autologous irradiated PBMC transiently expressing pp65 (4:1), and 5U/mL rhIL-2 (Chiron, Emeryville, CA) once a week for three weeks. 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 World Health Organization (WHO) established guidelines (Table S1), were obtained from patients in accordance with Institutional Review Board-approved protocols. Minced tumor specimens were implanted s.c. in the flank of NOD-*scid* mice, and remaining tumor was dissociated into single cells using 400 U/mL Collagenase III (Sigma-Aldrich, St. Louis, MO) in neural stem cell media (DMEM:F12 (Irvine Scientific); 1:50 B27 (Invitrogen, Carlsbad, CA); 5 μg/mL Heparin (Abraxis Pharmaceutical Products, Schaumburg, IL), 2 mM L-glutamine (Irvine Scientific) (12,13). Tumor spheres (TS) were expanded from either dissociated s.c. xenografts or primary tumor cells in neural stem cell

medium supplemented with 20 ng/mL EGF (R&D Systems, Minneapolis, MN), 20 ng/mL bFGF (R&D Systems), and 20 ng/mL LIF (Millipore, Billerica, MA) replenished in the culture medium twice a week. TS were dissociated with accutase (Innovative Cell Technologies, San Diego, CA) and differentiated in DMEM:F12, 2 mM L-glutamine, 25 mM HEPES, 7% FCS for 7-14 days. U251T glioblastoma adherent cells (gift from Dr. Waldemar Debinski, Pennsylvania State University) were grown in DMEM (Irvine Scientific) supplemented with 10% FCS, 2 mM L-glutamine, and 25 mM HEPES.

DNA Constructs

The CMVp:EGFP-ffLuc_pHIV7 lentiviral construct encodes an engineered fusion between enhanced green florescent protein (EGFP) and firefly luciferase (ffLuc) separated by a three glycine linker (EGFP:ffLuc) expressed under the control of the CMV-1 enhancer/promoter. The EF1p:pp65-2A-eGFP:ffluc_pHIV7 lentiviral vector encodes for EGFP:ffLuc, and CMV pp65 (gift from Dr. John Zaia, COHNMC) 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 (COHNMC). Construct sequences are provided upon request.

Flow cytometric analysis

Cell-surface phenotypes were assayed as previously described (15) using either fluorescein isothiocyanate (FITC)- conjugated anti-CD31, anti-CD45, anti-CD54, anti-HLA-DR, or anti-HLA-ABC (BD Biosciences, Jose, CA), or phycoerythrin (PE)-conjugated mouse anti-human CD133/1 and anti-human CD133/2 (Miltenyi Biotec, Bergisch Gladbach, Germany). Percent of immunoreactive cells was calculated using the subtraction method via FCS Express version 3 software (De Novo Software, Los Angeles, CA).

Protein analysis

Western blots were probed with rabbit polyclonal anti-Actin (Rockland, Gilbertsville, PA); goat polyclonal anti-Olig2 (R&D Systems), and mouse monoclonal anti-ß-III Tubulin (Millipore), anti-CD133 (Miltenyi), and anti-GFAP (Sigma-Aldrich) antibodies as per the manufacturers' instructions. Blots were imaged on the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) and band intensities were quantified using Odyssey v2.0 software (LI-COR).

Cytotoxicity and Cytokine assays

4-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 media at a final concentration of 10 μg/mL peptide for 2 h at 37°C. For concanamycin (CMA) inhibition, CTL were resuspended at 10^6 cells/mL and incubated for 2 hr at 37° C in the absence or presence of CMA (Calbiochem® EMD Chemicals, Inc., Gibbstown, NJ) prior to co-culture with tumor cells at a 10:1 effector:target (E:T) ratio.

CD107a degranulation assays were performed as described (17). For the flow based cytotoxicity assay, tumor cells were stained with 0.1 μM CFSE as per manufacturer's instructions (CellTrace™ CFSE Cell Proliferation Kit, Molecular Probes, Eugene, OR), peptide loaded, and then stained with APC-conjugated anti-CD133/1 and anti-CD133/2 (Miltenyi). The plates were incubated for 4 hr at 37°C, harvested and re-suspended with calibrite beads (1 drop/10 mL; BD Biosciences) and PI (0.5 μg/mL). Samples were run on the Cyan flow cytometer (Dako, Carpinteria, CA) and events collected were limited to a constant number of beads. Cytotoxicity was calculated based on the number of viable ($CSFE^+$ PI^-) tumor cells that were present at various E:T ratios and normalized to that obtained with control a CD19-specific CTL line.

Cytokine production was measured by co-culturing T cells with tumor at a 10:1 E:T ratio. After 20-24 hr incubation, supernatants were harvested and assayed using Luminex multiplex bead technology (Upstate, Waltham, MA), and output data were analyzed via the Bio-Plex Manager 4.0 (BioRad, Hercules, CA).

Tumor xenografts and *in vivo* biophotonic imaging

Mice were maintained under specific pathogen-free conditions, and all procedures were performed with 6-8 week old NOD-*scid* mice as approved by the COH Institute Animal Care and Use Committee. Intracranial tumor xenografts and biophotonic imaging were performed as previously described (12).

Immunohistochemistry

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

Results

Tumor spheres expanded from glioma explants exhibit stem cell-like characteristics

We employed two independent methods to expand short-term tumor sphere (TS) cultures of brain tumor stem/initiating cells (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 subcutaneous (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, 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 (Table S2), confirming 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 between 3-11% (Table S3). PBT003, 008 and 009 TS 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), upon subsequent expansion (> 6 passages) this line grew primarily as an adherent monolayer with a concomitant down-regulation 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 TS exhibit stem cell-like properties as judged by expression of established stem cell markers. TS cells express significant, though varying, levels of CD133 (Fig. 1A), ß-Catenin, BMI1, Nestin, SOX2 (Fig. S1) and OLIG2 (Fig. 1B). In contrast, when the TS 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, B). Moreover up-regulation of the astrocytic marker GFAP and the neuronal marker ß-III tubulin (Fig. 1B) was observed for all TS lines except PBT009 upon serum-differentiation. 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 TS secrete 10-30 fold higher levels VEGF as compared to differentiated cultures (Fig. 1C) (19).

To evaluate their *in vivo* tumorigenicity, TS 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 10^2-10^3 (Fig. 1D, Table S3; Fig. S2A-B). As exemplified by PBT003 TS, orthtopic tumors displayed similar histological features to the primary tumor with many common diagnostic features of glioblastoma (Fig. 1D). Cells derived from PBT008 however, showed reduced tumorigenicity as compared to the other expanded TS cultures, with mice remaining asymptomatic at 5-7 months and only a modest number of diffuse tumor cells being detected at this end point by IHC (data not shown). Such differences in tumor initiating potential and engraftment characteristics between tumor specimens has been reported by others (9). In summary, TS expanded for this study exhibit glioma cancer stem/initiating cell phenotypes, expression profiles, 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 down-regulating 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 $CD133^+$ tumor spheres, and $CD133^-$ serum-differentiated lines express significant and comparable levels of MHC I (HLA-A, -B, -C) and ICAM-1/CD54, suggesting 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 TS with expression levels decreasing upon 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.

We sought to verify our findings on non-cultured primary glioma specimens, and similar to Anderson et al. (24) found that all high-grade glioma specimens expressed detectable, but heterogenous 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 since freshly dispersed primary tumor samples did not contain significant numbers of $CD45^+$ cells (Fig. S3). To specifically evaluate MHC and ICAM-1 levels on the putative BTSC population, we co-stained 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 immunological 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 non-cultured high-grade glioma specimens have been reported (24,25), our results demonstrate that the $CD133^+$ BTSC population is not deficient in MHC I/II and ICAM-1 expression when compared to 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 impact 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 (Fig. S4A, B). Two cytolytic assays were employed in these experiments: 1) a luciferase-based cytolytic assay (LCA) (16) that reads out cell death based on decreases in cellular ATP and O_2 levels, and 2) a ^{51}Cr-release assay (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 12.2% ± 5.7% with killing of TS ≥ DIF lines) (Fig. 3A, B). T cell activation dependent cytokine production of IFN-γ and TNF-α was also shown to be similar upon engagement of either BTSC or differentiated targets (Fig. 3C), even though expression levels of co-stimulatory molecules such as NKG2D- and CD28-ligands differed between U251T and the PBT008 lines (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 CD133^{high} versus CD133^{low} 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 (Fig. S4C). Here too, we observed that an HLA-A24-restricted CMV-pp65 specific T cell clone (TCR$^+$, CD3$^+$, CD8$^+$, perforin$^+$, granzyme$^+$; Fig. S4D) killed with comparable efficiency both the BTSC and differentiated targets loaded with the CMV pp65 HLA-A24 binding peptide QYDPVAALF (average difference in cytotoxicity at all E:T ratios 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 co-cultured 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 co-stimulatory ligands (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 pre-formed lytic vesicles containing perforin and granzymes (27). Cell surface expression of the lysosomal-associated membrane proteins (LAMPs) 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 tumor cells that had been loaded with pp65 peptide, CD8$^+$ T cells degranulate equally as assessed by cell surface mobilization of CD107a (Fig. 4C). Concanamycin A (CMA), an inhibitor of vacuolar-type H$^+$ ATPase, is a highly specific inhibitor of perforin-dependent cytotoxicity 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 nM and greater (Fig. 4D). We therefore conclude that glioma CD133$^+$ TS and CD133$^-$ differentiated tumor populations are equally sensitive to CTL-mediated cytolysis involving granule exocytosis and perforin.

Glioma CD133$^+$ TS and CD133$^-$ tumor populations can process and present antigen for CD8$^+$ CTL recognition

The experiments that examine the sensitivity of glioma tumor cell subpopulations to CTL recognition upon exogenous addition of peptide antigen 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 containing CMV pp65 and a fusion of the EGFP and firefly luciferase (ffLuc) reporter genes, separated by the 2A self-cleaving peptide (Fig. 5A) (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 CTL 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 12.3% ± 8.2%) (Figs. 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 TS and pp65-specific CTLs were co-injected 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, since PBT003 is highly tumorigenic at doses as low as 1000 cells (Table S3), and 2×10^5 PBT003 cells were injected i.c. for these assays. As shown in Figure 6, co-injection of CMV-specific CTL with pp65-expressing PBT003 scp4 TS ablated the tumor initiation activity of antigen positive PBT003 BTSCs (i.e., all EGFP:$ffLuc^+$ $pp65^+$ cells). The specificity of this system was demonstrated 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 engrafted (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. Since tumor initiation is a hallmark feature of the CSC population, the inability of antigen-expressing PBT003 TS to initiate tumors when co-injected with antigen-specific CTL demonstrates that T cells are capable of efficiently eradicating the tumor initiating population of malignant glioma.

Discussion

The cancer stem cell (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 NK mediated lysis (22,23). We find evidence of equal expression of surface markers involved in immunological synapse formation, such as MHC I and the cell adhesion molecule ICAM. Likewise, IFN-γ mediated upregulation of MHC I on gliomas has also been shown to be comparable for CD133-positive and CD133-negative populations (23). We further demonstrate that BTSCs can process and present cytosolic protein antigens for MHC I for recognition by $CD8^+$ CTL. Moreover, we demonstrate that $CD8^+$ CTL can eliminate all cellular populations capable of tumor engraftment.

In this study we have employed 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 utilized 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 vial 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 continues to evolve, and for this reason our study did not rely on a single cell source or marker to define this population – i.e., based on TS growth, CD133 expression, and tumor-initiating activity, we found no deficiency in the targeting of these cells by CTL. TS expanded in serum-free media 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 media (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 TS represents an important step in the development of brain tumor models that more closely mimic the pathology and therapeutic response of the primary tumor.

Since glioma derived TS 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, CTL are capable of surveying the CNS parenchyma (34), and progress has been made towards exploiting CTL surveillance in a therapeutic context. Our group has shown that i.v. administered CTL 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 by FHBG PET imaging of T cells expressing HSV-TK 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 towards 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-based targeting of brain tumors are well spent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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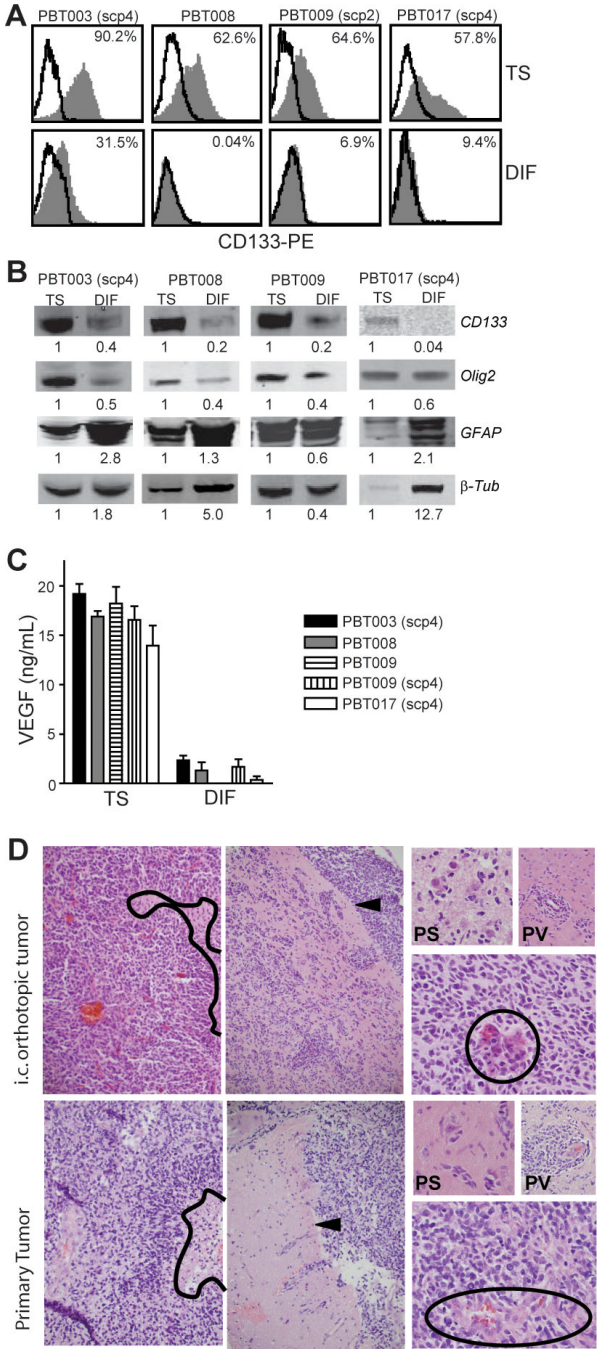

Figure 1. *In vitro* characterization of expanded tumor spheres and serum-differentiated cells expanded from primary high grade glioma specimens

(A) TS and DIF cells were analyzed by flow cytometry using anti-CD133 (grey) or isotype control antibody (solid line), and percent $CD133^+$ cells are indicated. (B) Western blots detecting CD133, Olig2, GFAP and ß-III tubulin in TS and DIF cells. Fold change in expression under serum growth conditions was normalized to actin and is indicated. (C) Quantitation (ng/ml) of VEGF secreted by TS and DIF cells. (D) Comparison of PBT003 scp4 TS orthotopic tumor histology (top panels) with primary human patient tumor histology (bottom panels) showing similar phenotypic appearance, with intrinsic diagnostic features of glioblastoma, including pseudopalisading necrosis (left panel outlines), prominent microvasculature (circle

and oval); and similar interactions with non-neoplastic host tissue elements, such as perineuronal satellitosis (PS), perivascular growth (PV), and marked leptomenigeal invasion (arrows).

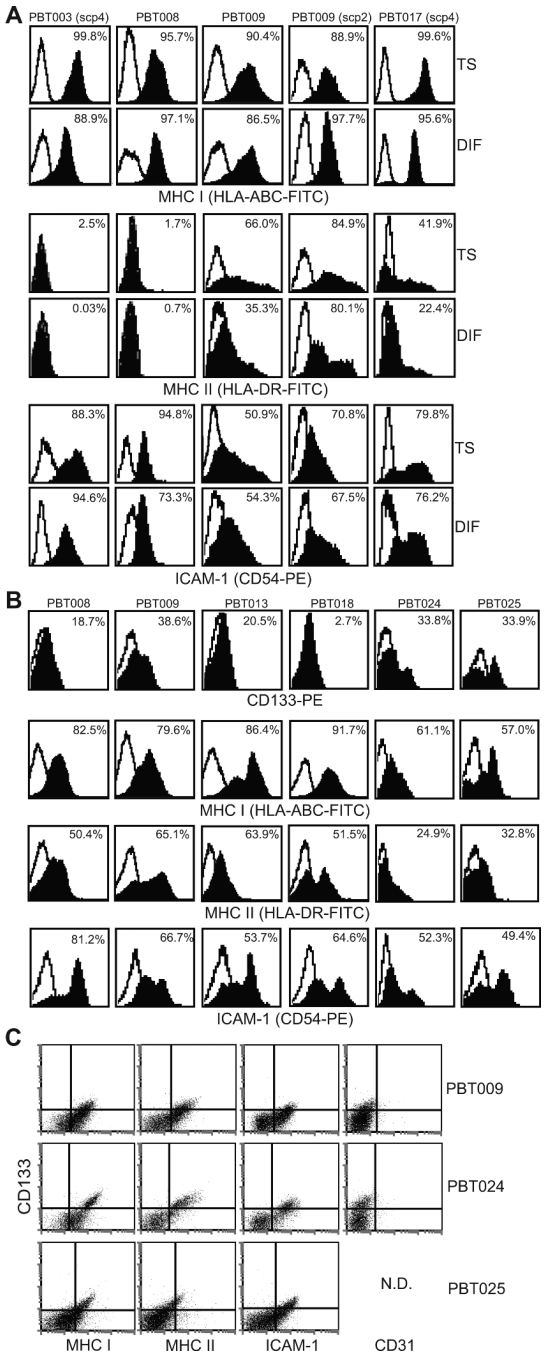

Figure 2. Flow cytometric analysis of MHC I, MHC II, and ICAM-1 expression

(A) TS (top panels) and DIF cells (bottom panels) and (B) freshly dispersed glioma tumors (FDT) were analyzed by flow cytometry using the indicated antibodies (black histograms) or isotype controls (solid line). Percent positive cells are indicated. (C) FDT cells were double stained for CD133 versus HLA-ABC, HLA-DR, CD54 (ICAM-1), or CD31 expression. N.D. not done.

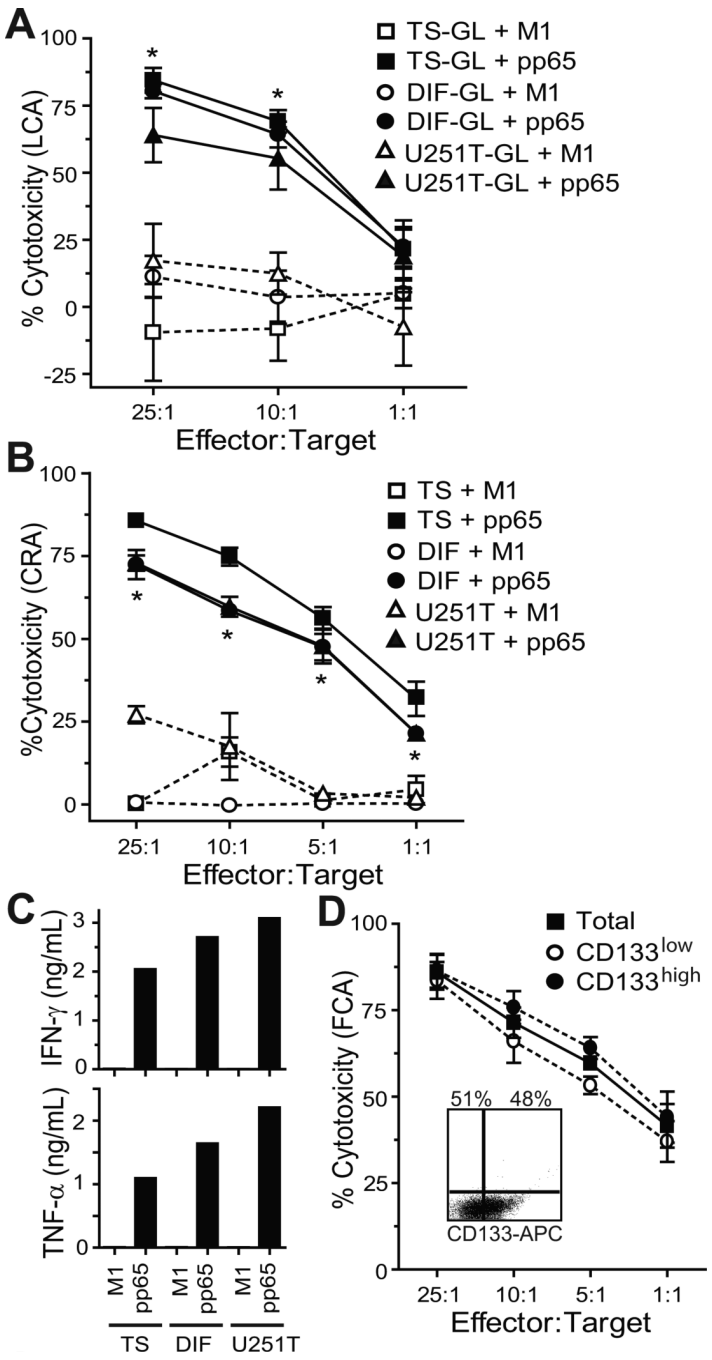

Figure 3. CMV-specific CTL kill pp65-peptide loaded PBT008 tumor stem cells equivalent to differentiated and established glioma cell lines

(A) LCA and (B) CRA measuring the lysis of CMV pp65 peptide (NLVPMVATV) versus control influenza M1 peptide (GILGFVFTL) loaded PBT008-GL$^+$ (EGFP-ffLuc$^+$) TS, serum-differentiated PBT008-GL$^+$ (DIF), or U251T-GL$^+$ cells by CD8$^+$ CMV pp65-specific CTL at increasing E:T ratios (mean ± S.D. of n=6 wells). *, $p < 0.006$ when comparing M1 versus pp65 peptide loaded targets using unpaired Student's t-test. (C) IFN-γ and TNF-α produced by CTL after co-culture with specified peptide loaded tumor targets. (D) Flow cytometry-based killing assay monitoring the loss of viable CD133^{high}, CD133^{low}, or total PBT008 TS cells at

increasing E:T ratios. Inset shows PBT008 TS CD133 expression, and the percent $CD133^{high}$ (48%) and $CD133^{low}$ (51%).

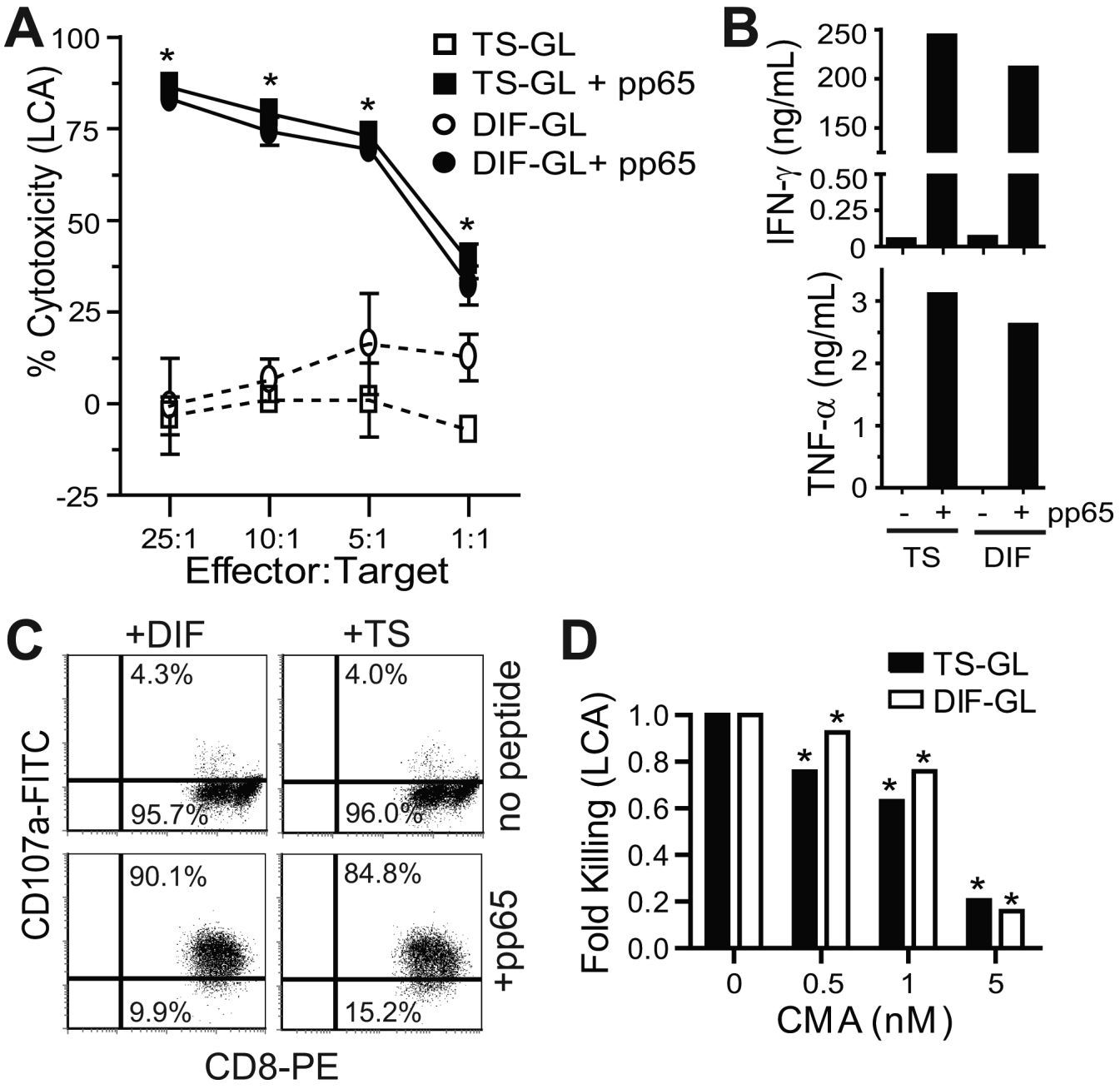

Figure 4. Perforin-dependent killing by CMV-specific CTL equivalently targets pp65-peptide loaded PBT003 TS and DIF cells

(A) Lysis of CMV pp65 peptide (QYDPVAALF) loaded PBT003-GL$^+$ (EGFP-ffLuc$^+$) TS or DIF cells by the CD8$^+$ pp65-specific CTL at increasing E:T ratios was determined by LCA (mean ± S.D. of n=6 wells). *, $p < 0.0004$ when comparing +/− pp65 peptide using unpaired Student's t-test. (B) IFN-γ and TNF-α produced by CTL after co-culture with specified tumor targets. (C) Flow cytometric detection of CD107a on CD8$^+$ CTL after co-culture with tumor cells as in (A) at a 2:1 E:T ratio in the presence (lower panels) and absence (upper panels) 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 upon pre-incubation of CD8$^+$ pp65-specific CTL

with increasing concentrations of CMA. At all CMA concentrations $p < 0.003$ when compared to no CMA using unpaired Student's t-test.

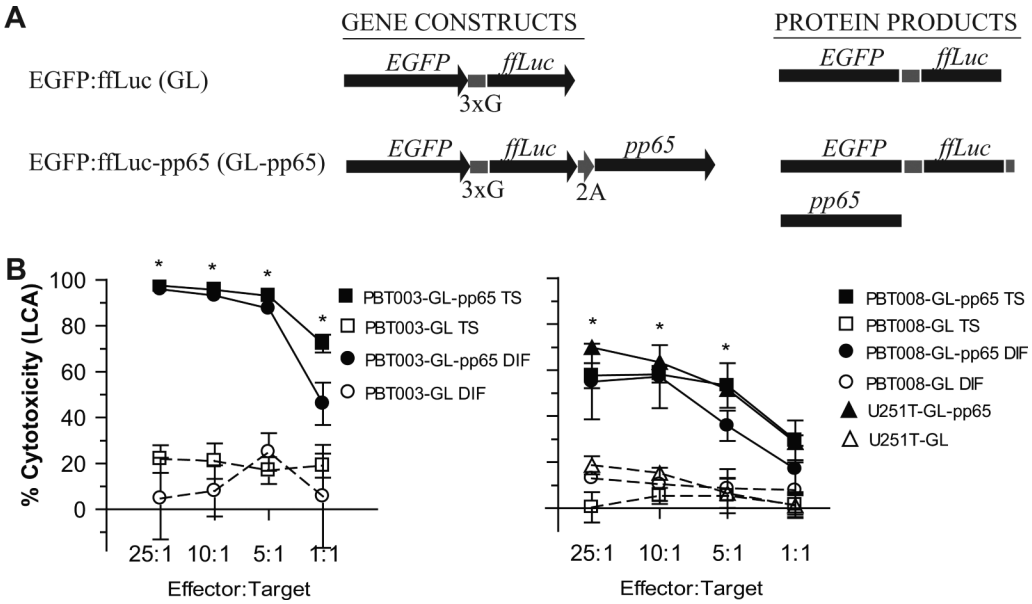

Figure 5. Tumor stem cells and serum-differentiated glioma cells expressing endogenous pp65 antigen are equivalently recognized and lysed by CD8$^+$ CTL

(A) Schematic of EGP:ffLuc-2A-pp65 bicistronic construct endowing forced co-expression of the EGFP:ffLuc reporter and pp65 antigen. 3×G, 3 glycine linker; 2A, self-cleaving peptide. (B) Lysis of tumor cells expressing either EGFP:ffLuc alone (GL) or EGFP:ffLuc and the CMV-pp65 antigen (GL-pp65) by HLA-matched CD8$^+$ CMV-specific CTL was determined by LCA (mean ± S.D. of n=6 wells). *, $p < 0.045$ when comparing GL to GL-pp65 expressing targets using unpaired Student's t-test.

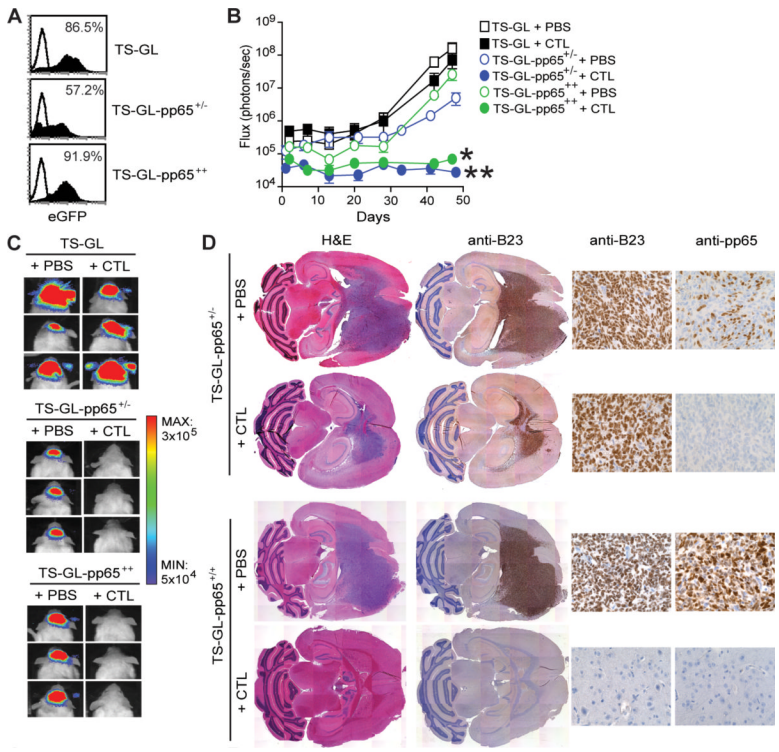

Figure 6. CMV-specific CTL ablate the tumor initiation potential of pp65 expressing TS

2×10^5 PBT003 (scp4) TS expressing either EGFP:ffLuc (TS-GL) or EGFP:ffLuc and the CMV-pp65 antigen (TS-GL-pp65) were co-injected 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, 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) Mean biophotonic flux of the i.c. tumors ± S.E. over time was determined by Xenogen imaging. *, p = 0.017 when comparing TS-GL-pp65$^{+/+}$ tumors that had been co-injected with CTL vs. PBS at day 47 using an unpaired Student's t-test. **, p = 0.036 when comparing TS-GL-pp65$^{+/-}$ tumors that had been co-injected with CTL vs. 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 panels) or with CTL (bottom panels) and were stained with H&E (left), or for the B23 human cell marker (middle two) or the CMV pp65 antigen (right) by IHC. Anti-B23 and anti-pp65 IHC near the injection site is shown to the right.