CMV-Independent Lysis of Glioblastoma by Ex Vivo Expanded/Activated Vδ1+ γδ T Cells

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Abstract

Vδ2neg γδ T cells, of which Vδ1+ γδ T cells are by far the largest subset, are important effectors against CMV infection. Malignant gliomas often contain CMV genetic material and proteins, and evidence exists that CMV infection may be associated with initiation and/or progression of glioblastoma multiforme (GBM). We sought to determine if Vδ1+ γδ T cells were cytotoxic to GBM and the extent to which their cytotoxicity was CMV dependent. We examined the cytotoxic effect of ex vivo expanded/activated Vδ1+ γδ T cells from healthy CMV seropositive and CMV seronegative donors on unmanipulated CMV-infected established GBM cell lines and cell lines developed from short-term culture of primary tumors. Expanded/activated Vδ1+ T cells killed CMV-negative U251, U87, and U373 GBM cell lines and two primary tumor explants regardless of the serologic status of the donor. Experimental CMV infection did not increase Vδ1+ T cell-mediated cytotoxicity and in some cases the cell lines were more resistant to lysis when infected with CMV. Flow cytometry analysis of CMV-infected cell lines revealed down-regulation of the NKG2D ligands ULBP-2, and ULBP-3 as well as MICA/B in CMV-infected cells. These studies show that ex vivo expanded/activated Vδ1+ γδ T cells readily recognize and kill established GBM cell lines and primary tumor-derived GBM cells regardless of whether CMV infection is present, however, CMV may enhance the resistance GBM cell lines to innate recognition possibly contributing to the poor immunogenicity of GBM.

Introduction

High-grade gliomas such as glioblastoma multiforme (GBM) can initiate and progress to an unsalvageable point without generating a significant immune response, consistent with Medawar’s description of the brain as a site of relative immune protection [1]. Human cytomegalovirus (HCMV) infection has also been detected in a large percentage of human high-grade gliomas, and recent studies suggest a relationship between HCMV infection and initiation and/or progression of GBM [2–6]. The presence of latent CMV infection in GBM could present an opportunity for CMV-based immunotherapy, provided that such an approach could overcome the highly immunosuppressive microenvironment [7–11].

T cells bearing the γ and δ receptor (γδ T cells) are important effectors against malignancy-associated viral infections such as EBV [12] and HSV [13]. Indeed, increases principally in circulating Vδ1+, and to a lesser extent Vδ3+ and Vδ3+ T cell subsets [14], have been strongly and positively correlated with a response to and subsequent resolution of HCMV viremia [15]. Most importantly, CMV-reactive Vδ1+ γδ T cells also are cross-reactive against several malignant cell lines [15–18].

The Vδ1 subset is normally <10% of circulating γδ T cells but predominant in epithelial tissues. Vδ1+ T cells are activated by stress-induced self-antigens such as MIG-A/B and UL-16 binding proteins through the T cell receptor and NKG2D ligands ULBP-2, and ULBP-3 as well as MICA/B in CMV-infected cells. These studies show that ex vivo expanded/activated Vδ1+ γδ T cells readily recognize and kill established GBM cell lines and primary tumor-derived GBM cells regardless of whether CMV infection is present, however, CMV may enhance the resistance GBM cell lines to innate recognition possibly contributing to the poor immunogenicity of GBM.
mediated immune reactivity against GBM, and the extent to which CMV infection in high-grade gliomas affects their immunogenicity to V\(\delta^+\) T cells.

**Materials and Methods**

Patients and healthy volunteers

Patients presenting with CT or MRI evidence of probable GBM were accrued for this study and enrolled following histological diagnosis. Patients and controls were excluded if they had been diagnosed with a co-existing immune system disorder; active viral, bacterial or parasitic infection; or prior organ or bone marrow transplant. The University of Alabama at Birmingham (UAB) Institutional Review Board for Human Research approved this study. Written informed consent was obtained from each patient or a designated family member. Written informed consent was obtained from healthy volunteers following explanation of the research studies.

Expansion of Peripheral Blood \(\gamma\delta^+\) T cells and tumor-infiltrating lymphocytes

Two methods were used to expand peripheral blood \(\gamma\delta^+\) T cells from healthy volunteers and GBM patients \((n = 5\) / group), one that preferentially expands the V\(\delta^V\)82 cell population (ZOL/IL-2) and another that inhibits apoptosis in all V\(\delta^+\) T cells. In the ZOL/IL-2 method, mononuclear cells (MNC) were obtained by density gradient centrifugation and resuspended at \(1.0 \times 10^6\) cells/ml in RPMI 1640 supplement with L-glutamine and 10% human serum. V\(\delta^+\) T cell subsets (CD2/OKT-3) were determined by density gradient centrifugation and resuspended at 1.0 \(\times 10^6\) cells/ml in RPMI 1640. 10% pooled human serum. V\(\delta^+\) T cells from healthy volunteers and GBM patients \((n = 5\) / group) were expanded preferentially using recombinant-human (rHu) IL-2 added to the culture media for 5 days, 10 ng/ml anti-CD3 mAb OKT3 (Ortho Biotech) and 300 U/ml IL-2 (Roche; Indianapolis, IN). The culture was maintained at the original density for 14 d with addition of 50 U/ml IL-2 on post-culture days 2, 6, and 10, and 10 and addition of complete media as determined by pH and cell density. In the CD2/OKT-3 method described by Lopez [37], cultures were initiated in RPMI-1640 supplemented with L-glutamine, and 10% human serum 10% 1 M HEPES, 1,000 U/ml human rIFN-\(\gamma\), 10 U/ml human IL-12, and 1 \(\mu\)g/ml mouse anti-human CD2 mAb clone CLB-CD2 6G4 (Baxter Oncology, Deerfield, IL). Twenty-four hours later, 10 ng/ml anti-CD3 mAb OKT3 (Ortho Biotech) and 300 U/ml recombinant-human (rHu) IL-2 were added to the culture media and maintained at the original density for 14 d with addition of complete media as determined by pH and cell density. Tumor-infiltrating lymphocytes cultures were initiated from fresh GBM tumors from operative specimens. Tissues were minced finely with #11 scalpel blades followed trypsin and collagenase type IV for 2 h at room temperature. After digestion, the cells were washed twice in RPMI1640 and cultured using only the CD2/OKT-3 method as described above and maintained for 14 d with the addition of fresh complete media as needed.

Expansion of peripheral blood V\(\delta^+\) T cells from healthy volunteers

Up to 50 ml of peripheral blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and resuspended at a concentration of 1 \(\times 10^6\) cells/ml in RPMI-1640 supplemented with 10% pooled human serum. V\(\delta^V\)1+ T cells were sorted from PBMC using anti-V\(\delta^1\) (R9.12 Beckman Coulter) and ferromagnetic particle (Miltenyi Biotech; Bergchen Gladbach, Germany). Purified V\(\delta^1\) T cells were expanded in 200 IU/ml recombinant IL-2 (R&D Systems; Abingdon, UK), 1 \(\mu\)g/ml PHA-L (Sigma-Aldrich; Taufkirchen, GE) and irradiated allogeneic PBMC feeder cells. After 3 weeks of culture, the resulting effector lines were phenotyped and purity (routinely >95%) was determined by multicolor fluorescent staining.

**Cell Lines**

U251, U87, and U373 are established GBM cell lines that were obtained from the Brain Tumor Tissue Facility at UAB and have been verified to be authentic by short-tandem repeat PCR conducted by the UAB Cancer Genomics Core Facility (Michael Crowley, Director). The U87 is a grade IV glioma that originated from a 44-year-old Caucasian woman [38]. The genetic characteristics of the cell line have been well described [39]. The UAB Brain Tumor Tissue Core, a unit of the UAB NCI SPORE in Brain Cancer, obtained the cell line from the ATCC. Its origin has been verified by STR PCR and has been found to agree with the original cell source. U251MG is a grade III astrocytoma that was cultured from a 61 year old male [38] and was obtained directly from Darel D. Bigner (Duke University) who obtained them from Jan Ponten of Uppsala University. The cell line has been verified as authentic (Rb-deleted, p15/p16 wild type) and has the same STR pattern as the original line. U373MG has been previously identified as a subclone of the U251 cell line [40]. Cell lines were cultured in a 50:50 mixture of Dulbecco's Minimum Eagle's Medium and Ham's Nutrient Mixture F-12 (DMEM/F12 50:50) enriched with 7% heat inactivated pooled human cGMP-grade serum (Labquip; Woodbridge, ON), 2 mM L-glutamine and 10% Penicillin-Streptomycin-Amphotericin (Mediatech; Herndon VA). The human fetal lung fibroblast line MRC5 was obtained from European Collection of Cell Cultures (ECACC). Cells were cultured in MEM supplemented with 2 mM L-glutamine, nonessential amino acids (all from Sigma Aldrich) and 10% FCS. Fibroblasts were used between 32-40 passages and were maintained at 37°C in a humid atmosphere containing 5% CO2.

**Primary Tumor Explant Cultures**

Short-passage primary GBM cultures were initiated from intraoperative specimens obtained from accrued patients. Tissue for explant was transferred aseptically into T75 flasks in about 15 ml of “complete culture medium” which is DMEM/F12 50:50 (Mediatech; Herndon VA) supplemented to 2 mM L-glutamine, nonessential amino acids (all from Sigma Aldrich) and 10% FCS. Fibroblasts were used between 32-40 passages and were maintained at 37°C in a humid atmosphere containing 5% CO2.

**Immunohistochemistry**

Paraffin embedded sections of primary GBM were used for immunohistochemistry. Antibodies and sources were as follows: HCMV IE-1 (Millipore: Billerica, MA), MIC A/B (Bio Legend; San Diego, CA), ULBP-1, ULBP-2, ULBP-3, and ULBP-4 (Santa Cruz Biotechnology; Santa Cruz, CA). Sections of normal brain were deparaffinized in xylene and rehydrated by ethanol washes at decreasing concentrations (100%, 95%, 70%) followed by PBS. Boiling the sections for 5 min with EDTA in the pressure cooker was performed for antigen retrieval. Endogenous peroxidase quenching was performed with 3% peroxide for 5 min and rinsed with Tris buffer. 3% normal goat serum was then applied as a blocking agent for 20 min. Primary antibody was applied as a blocking agent for 20 min. Primary antibody was applied and the sections were washed for 5-7 days and any cells growing out from the explants were maintained in primary culture. The cultures were monitored for growth and confluence and the media was changed weekly. Once cells on the flask were confluent, the cells were detached using trypsin and passaged and/or cryopreserved.
20 min. Diaminobenzidine tetrahydrochloride (DAB) (Biogenex, San Ramon CA) was then applied for 8 min. After DAB staining, slides were rinsed with DI water and counterstained with pure hematoxylin for 45 s. Then the slides were dehydrated with graded ethanol and xylene and mounted in coverslips.

In situ hybridization

For detection of HCMV nucleic acids, a biotinylated 21-base oligonucleotide (5′-GTGGTGGCGCTGGGGGTGGCG-3′) specific for HCMV early gene mRNA and biotinylated positive (specific for polyadenylic mRNA) and negative control (specific for HSV-1/2) probes were obtained (Novocastra; Buffalo Grove, IL). We performed enzyme digestion and nucleic acid denaturation of paraffin sections using a Misha thermocycler (Shandon Lipshaw, Pittsburgh, PA), and slides were hybridized overnight at 37°C in a humidified chamber (methods are detailed in manuscript in preparation). 4 Probe was detected using a supersensitive detection system (BioGenex, chromogen NBT). To detect HCMV DNA, we used a digoxigenin-labeled HCMV total genome DNA probe (Zymed Labs, South San Francisco, CA). The manufacturer provided positive (specific for endogenous alu DNA sequence) and negative (nonspecific DNA) digoxigenin-labeled control probes.

CMV strains, infection procedure

Both clinical CMV strains TB40/E and VHL/E were kindly provided by Dr. Christian Sinzger (University of Tubingen, Germany). For infection, 5 × 10⁵ MRC5 fibroblasts per well were seeded into 24-well plate 24 h before adding the virus. Subconfluent monolayers of MRC5 fibroblasts were incubated with CMV in suspension at multiplicity of infection (MOI) of 1–5 for 2 h at 37°C. After virus adsorption, cells were washed and cultured for 2–4 days. Infection was verified by microscopy to determine the cytopathic effect.

Cytotoxicity assays

Targets are labeled with the membrane dye PKH26 (Sigma; St. Louis, MO). Expanded/activated γδ T cells are then added to the tubes at ratios of 0:1 (Background), 5:1, 10:1, 20:1 and 40:1 effectors/GBM targets, incubated for four hours at 37°C and 5% CO₂, washed × 1 and resuspended in 1 ml HBSS. To-Pro Iodide solution (Molecular Probes; Eugene, OR) 20 μl is added prior to acquisition on the flow cytometer. Cytotoxicity is calculated by dividing the number of PKH26+To-Pro Iodide+ events by the total number of PKH26+ events and multiplying the result by 100.

Statistical methods

Descriptive statistics and nonparametric analysis using the Wilcoxon log-rank test was used to test for differences in expansion of γδ T cells observed between healthy donors and GBM patients and differences cytotoxicity of Vδ1+ T cells between CMV+ and CMV− donors. Where applicable, paired-t tests were used to compare cytotoxicity data and NKG2DL expression (data normally distributed).
Peripheral blood γδ T cells obtained from patients prior to treatment and GBM-infiltrating γδ T cells obtained from resected tumor show minimal response to ex vivo stimulation.

We previously reported that the proportion of circulating Vδ1+ T cells is increased in GBM patients and that the absolute Vδ1+ T cell count did not significantly differ from older healthy volunteers [36]. In these experiments, we cultured peripheral blood mononuclear cells (MNC) from five GBM patients in an effort to determine whether the Vδ1+ T cells could respond to ex vivo stimuli and whether sufficient numbers of cells could be obtained for cloning and assay for anti-CMV activity. As shown in Figure 1, γδ T cells from patients with GBM failed to expand using either the ZOL/IL-2 or CD2/OKT-3 culture conditions. Less than 5-fold expansion was seen in all peripheral blood specimens from GBM patients by both methods, neither of which showed a significant advantage in expanding Vδ1+ T cells from GBM patients (p = 0.12). In contrast, γδ T cells from healthy volunteers expanded well using CD2/OKT-3 (n = 7, median = 200 with range 100 to 375 fold) or ZOL/IL-2 (n = 5, median = 223 fold, range 46 to 435 fold). Few γδ T cells were seen in tumor infiltrating lymphocytes (TIL) preparations obtained from freshly resected GBM tumors and these also did not respond to T cell stimulation culture. (Figure 2).

Vδ1+ T cells from CMV-seronegative and CMV-seropositive healthy volunteers lyse standardized glioma cell lines and short-passage primary explants from GBM tumors equally well

Vδ1+ T cells from CMV- (Donor 1) and CMV+ (Donor 2) healthy volunteers cultured using a standardized culture method developed in our laboratory [18] consistently yielded over 90% Vδ1+ T cells (Figure 3a) and efficiently lysed glioma cell lines U87, U251 and U373 and primary GBM explants designated 1016 and 1042 (Figure 3b and Figure s1). Expanded/activated Vδ1+ T cells were not cytotoxic to cultured human astrocytes in E:T ratios up to 20:1. Cytotoxicity against CMV-infected astrocytes was <5% at the highest E:T ratio (20:1) assessed. Additional experiments that incorporated cytolytic activity of Vδ1+ T cell cultures from CMV seropositive (n = 4) and CMV seronegative (n = 6) donors against U251 cells and the GBM primary explant line 1042 (Figure 3c) revealed no significant impact on cytotoxicity based on CMV serologic status (p = 0.75 and p = 0.29 respectively).

After establishing that Vδ1+ T cells are cytotoxic to GBM cells, we next considered whether this Vδ1+ T cell-mediated recognition and lysis of primary glioma explants could be associated with latent CMV infection. Indeed, the two primary GBM tumors from which cell explants 1016 and 1042 were derived expressed CMV-associated antigens IE-1 and pp65 (Figure 4a) as well as CMV mRNA (Figure 4b, upper panel). CMV mRNA was also present in the expanded Vδ1+ T cell cultures.
detectable in both cell lines (Figure 4b) suggesting continued CMV infection. As expected and previously reported, there was no evidence of deep parenchymal T cell invasion of these tumors (Figure 4a). Strong expression of V<sup>δ1</sup> T cell target NKG2D ligands (NKG2DL) ULBP-2 and ULBP-3 and moderate expression of ULBP-1 was noted on both tumor cell lines. There was little to no expression of MIC-A/B on either line (Figure 5).

Experimental acute CMV infection of selected glioma cell lines is associated with a reduction of NKG2DL expression and in some cases reduced cytotoxicity. In order to determine the impact of CMV infection on tumor vulnerability to V<sup>δ1</sup> T cells, we infected the CMV-negative glioma cell line U251 with CMV strain TB40/E and assessed V<sup>δ1</sup> T cell cytotoxicity against both the infected and unmanipulated cell lines. Expanded/activated V<sup>δ1</sup> T cells lysed both CMV-infected and unmanipulated U251 cells, although a significant reduction in cytotoxicity to CMV-infected U251 cells was noted at the E:T ratio of 20:1 (n = 6, p = 0.021). Figure 6a shows results of cytotoxicity assays against U251 from three representative donors. When examined separately, donor CMV serologic status (n = 3 seropositive and 3 seronegative) was not a factor in lysis of either infected or unmanipulated cells (p = 1.00 at 1:20 effector:target ratio, data not shown). Figure 6b compares NKG2DL expression between unmanipulated U251 cells and U251 cells that had been infected in culture for 5 days. MIC A/B and ULBP-2 were consistently down regulated in CMV-infected U251 cell cultures. ULBP-1 and ULBP-3 expression was variable between cultures. When CMV-infected U251 cells were assessed based on expression of HCMV-IE-1 expression (Figure 6c), however, it was noted that cells strongly expressing IE-1 (red histograms) generally showed little change in MIC-A/B expression when compared to a parallel unmanipulated culture (black histograms). Unmanipulated cells do not express IE-1 (data not shown) and expressed all UL-16 binding proteins at higher density than CMV-infected cells. Interestingly, the IE-1<sup>neg</sup> population (blue) showed only ULBP-2 expression. This population contains a mixture of fragile infected and non-viable cells many of which have shed these surface markers. Taken together, as V<sup>δ1</sup> T cells...
were resistant to myeloma [26,45,46]. When cells are obtained from patients with neuroblastoma and ex vivo expanded/activated V

have extensively shown persistent CMV infection. Clinical data and murine models both support a vigorous T cell response to cancer is currently limited, particularly with respect to glioblastoma tumors that have shown persistent CMV infection.

Strategies for expansion of circulating Vγ9Vδ2+ T cells have been elusive. Indeed, our laboratory and others have extensively reported poor persistence, death, and functional anergy of ex vivo expanded/activated Vγ9Vδ2+ T cells, particularly when they are obtained from cancer patients [33,42–44]. Ex vivo expansion of Vδ1+ γδ T cells, however, has met with some success, particularly when cells are obtained from patients with neuroblastoma and myeloma [26,43,46]. Vδ1+ γδ T cells GBM patients, however, were resistant to ex vivo stimulation methods that readily expand this population in healthy volunteers and appear to be functionally anergic.

We then tested the cytotoxicity of ex vivo expanded Vδ1+ T cells from healthy volunteers against standardized glioma cell lines and two cultured explants derived from primary brain tumors grown in short-term culture to determine if CMV infection was associated with vulnerability to recognition and lysis. The two primary tumor explants designated 1042 and 1016 were effectively lysed by ex vivo expanded Vδ1+ T cells (Figure 3 and Figure s1) and were shown to be positive for CMV infection both in sections from the primary tumors and the short-passage cell lines derived from them. We were able to demonstrate strong expression of pp65 and IE-1 as well as the presence of CMV mRNA in the primary GBM tumors from which the cell preparations were obtained. Cultured cells from these tumors also showed evidence of continued CMV infection (Figure 4) and expressed several stress-associated NKG2D ligands (Figure 5). However, Vδ1+ T cells also consistently lysed U251MG, U87MG, and U373 GBM cell lines, which do not express CMV-associated antigens nor do they harbor replicating CMV. These findings are consistent with earlier work from this laboratory that documents significant CMV-independent cytotoxicity of purified Vδ1+ T cells against leukemia and myeloma cell lines and primary leukemia [30,31,46].

Cytotoxicity of γδ T cells against tumor cells and virus-infected cell lines is broadly based and is generally related to expression of stress-associated surface proteins [19,47]. We have previously shown that γδ T cells with strong cytotoxicity to glioma cell lines do not kill normal human astrocytes in culture [48], and in this work we have also shown that CMV-infected astrocytes are also not killed by cytotoxic Vδ1+ T cells. Separately, we have also shown essentially negative cytotoxicity toward uninfected vs. strong cytotoxicity toward CMV-infected MRC fibroblasts [18]. Taken together, these data strongly suggest that Vδ1+ T cell

Discussion

In previous work we have shown that γδ T cell cultures cultured ex vivo using a combination of anti-CD2, OKT-3, and IL-2 are cytotoxic to high-grade gliomas in vivo and to human xenograft tumors in immunodeficient mice [34,35]. We now extend our previous findings to examine the specific function of the Vδ1+ T cell population in the context of the immune response and potential immunotherapies for malignant glioma. Clinical data and murine models both support a vigorous γδ T cell response to CMV infection [14,41] that is skewed toward Vδ1+ population is comprised principally of Vδ1+ T cells, a population with both cytotoxic and regulatory/suppressive roles in malignancy and infection [33]. Understanding of the function of Vδ1+ T cells in the immune response to cancer is currently limited, particularly with respect to glioblastoma tumors that have shown persistent CMV infection.

Figure 4. Assessment of CMV activity in primary GBM and derived cell lines. (a) Primary GBM from which cell explants 1016 and 1042 were derived expressed CMV-associated surface antigens IE-1 and pp65, although T cell invasion beyond perivascular areas as indicated by anti-CD3 labeling is minimal. (b) Both primary tumors 1016 and 1042 (top panel) were assessed for the presence of CMV mRNA by in-situ hybridization using a biotinylated 21-base oligonucleotide (5'-GTGGTGGCGCTGGGGGTGGCG-3') specific for HCMV early gene mRNA and a biotinylated positive (specific for polyadenylic mRNA) and negative control (specific for HSV-1/2) probe. provided positive (specific for endogenous alu DNA sequence) and negative (nonspecific DNA) digoxigenin-labeled control probes. Both tumors show the presence of CMV mRNA as do the short passage cell lines that were derived from them (bottom panel) suggesting continued CMV infection.
recognition and cytotoxicity against gliomas is at least in part modulated by expression of stress-induced NKG2DL and independent of latent or persistent CMV infection.

Although our findings suggest that Vδ1+ T cells were not dependent on CMV infection in order to recognize and lyse gliomas, Vδ1+ T cells potentially could recognize glioma cells based on previous exposure to CMV. This “crossover” cytotoxicity was first shown by Halary [16], who found that that Vδ2neg T cells (of which Vδ1+ T cells are the predominant population) both recognize and respond to CMV-infected fibroblasts and lye the HT-29 intestinal tumor cell line independent of CMV infection. Based on this previous work, we examined the cytotoxic function of expanded Vδ1+ T cells obtained from healthy CMV seropositive and seronegative donors against standardized glioma cell lines and against cells cultured from freshly resected GBM. We were unable to demonstrate any difference in cytotoxicity between Vδ1+ T cells expanded from either CMV+ or CMV− donors against the glioma targets (Figure 3). These findings are consistent with earlier work that found that although an expanded and clonally restricted Vδ1+ T cell population could be documented both in CMV-seropositive and CMV-seronegative healthy individuals, the cytolytic response to CMV-infected fibroblasts was no different between the donor groups [18].

Having established that Vδ1+ T cell-mediated recognition and lysis was not dependent on CMV infection, we then asked whether CMV infection could potentiate or inhibit Vδ1+ T cell recognition of gliomas. When standardized CMV-glioma cell lines were artificially infected with CMV, Vδ1+ T cell cytotoxicity against glioma cell line targets was either less or the same at comparable effector:target ratios (Figure 6). CMV infection did not result in increased vulnerability of GBM cell lines to Vδ1+ T cell mediated cytotoxicity. Inhibition of Vδ1+ T cell cytotoxicity to CMV-infected GBM could occur through several mechanisms. Paradoxically, down-regulation of NKG2D has been shown in NK cells when exposed to CMV infected glioma, a response thought to be associated with control of the immune response to virus CMV.
infection [49], as has the sequestration of NKG2DL on infected cells [50]. Blocking of MIC-A/B and individual UL-16 binding proteins decreases cytotoxicity of $\gamma\delta$ T cells to U251MG cells but do not abrogate cytotoxicity entirely [48]. Blocking of 4 NKG2DL simultaneously decreases cytotoxicity by approximately 40%, consistent with the reduced cytotoxicity seen in CMV-infected U251MG. Indeed, our findings detailed in Figure 6b shows modulation of MIC-A/B and ULBP-2 consistent with previous reports of UL-16 sequestration or modulation of expression in multiple NKG2DL ligands in CMV-infected cells. [51,52]. Taken together, these findings suggest that sequestration of NKG2DL in CMV-infected U251MG cells is a contributing factor to decreased immunogenicity of glioma cells to recognition by NK and $\gamma\delta$ T cells. These findings, however, must be interpreted in the context of experimental CMV infection, which is substantially different than the long-term latent CMV infection of GBM in situ. A further complication is the use of CMV strains that are propagated in the laboratory that may have critical differences from the CMV strains that have been isolated from GBM. Investigators have recently published an in vitro model of long-term CMV infection in a GBM cell line that can retain the virus in multiple passages which if reproducible and successful in animal models may reflect CMV interaction with GBM and the immune microenvironment with greater accuracy [53].

In summary, we have shown that pure cultures of expanded and activated allogeneic $\gamma\delta$ T cells are cytotoxic to primary $\gamma\delta$ T cells and in some cases will decrease immunogenicity by down-regulation of NKG2DL ligands expression. In addition, $\gamma\delta$ T cells from CMV-seropositive individuals do not show enhanced cross-reactivity to primary tumor explants or glioma cell lines as has been seen in other tumor types. These findings suggest that $\gamma\delta$ T cells are potent effectors against glioma, are not dependent of CMV infection for their cytotoxicity, and should be further explored in the design of immunotherapeutic strategies for high-grade brain tumors.

**Supporting Information**

Figure S1 Detail of Cytotoxicity of $\gamma\delta$ T Cells against glioma cell lines and primary GBM at a range of Effector:Target ratios from Figure 3b. (TIF)
Author Contributions
Conceived and designed the experiments: AK LSL WB GYG MWL. Performed the experiments: AK HA YS LH. Analyzed the data: LL AK GYG GC. Contributed reagents/materials/analysis tools: GYG. Wrote the paper: AK LL WB GYG.

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