Impact of Human Cytomegalovirus on Glioblastoma Cell Viability and Chemotherapy Treatment

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Keywords: Human Cytomegalovirus, glioblastoma multiforme, chemotherapy, Temozolomide, Carmustine.

Article type: Research Article; Subject category: Animal, DNA Viruses

Word count: 4484

Abbreviations: BCNU, Carmustine (bis-chloroethyl nitrosourea); BER, base excision repair; cmvIL10, HCMV interleukin 10 homolog; DMSO, dimethyl sulfoxide; DSBs,
DNA double-strand breaks; EtOH, ethanol; FACS, fluorescence-activated cell sorting; GBM, glioblastoma multiforme; HCMV, Human cytomegalovirus; hpi, hours post infection; MGMT, O6-methylguanine DNA methyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; STAT3, signal transducer and activator of transcription 3; TMZ, Temozolomide; PBS, phosphate-buffered saline; PI, propidium iodide.
The relationship between the Human Cytomegalovirus (HCMV) and tumors has been extensively investigated, mainly in glioblastoma multiforme (GBM), a malignant tumor of the central nervous system with low overall survival. Several reports demonstrated the presence of HCMV in GBM, although typically restricted to a low number of cells, and studies indicated that viral proteins have the ability to dysregulate cellular processes and increase tumor malignancy. Treatment of GBM involves the use of the chemotherapeutic agents Temozolomide (TMZ) and Carmustine (BCNU), which lead to attachment of adducts to the DNA backbone, causing errors during replication and consequent cell death. It is known that HCMV infection can modulate DNA repair pathways, but the effects the virus may exhibit during chemotherapy are unknown. Here we approach this question by analyzing HCMV infection and viral protein accumulation in GBM cell lines with different genotypes and their response to TMZ and BCNU in the presence of the virus. We demonstrate that A172, TP365MG and U251MG GBM cells are efficiently infected by both low passage (TB40E) and high passage (AD169) HCMV strains. However, the GBM cell lines vary widely in permissiveness to viral gene expression and exhibit very different patterns of immediate early, early and late protein accumulation. HCMV reduces viability of permissive GBM cells in a multiplicity-dependent manner both in the absence and presence of TMZ or BCNU. In sum, we demonstrate that GBM cell lines are equally susceptible but differentially permissive to infection by both low and high passage strains of HCMV. This observation not only indicates that viral replication is largely controlled by cellular factors in this system, but also provides a possible explanation for why viral gene products are found in only a subset of cells in GBM tumors.
Furthermore, we conclude that the virus does not confer increased resistance to chemotherapeutic drugs in various GBM cell lines but instead reduces tumor cell viability. These results highlight that the oncomodulatory potential of HCMV is not limited to cancer promoting activities but also comprises adverse effects on tumor cell proliferation or survival.
Human cytomegalovirus (HCMV) is a member of the herpesvirus family and is present in the majority of people worldwide, including almost 100% of the adult population in developing countries [1]. Congenital infection by HCMV is the most common cause of severe neurological impairments including hearing loss, optic atrophy and microcephaly in many parts of the world [1]. The virus is also a life-threatening pathogen in immunocompromised individuals such as transplant recipients as well as patients with AIDS or cancer [2].

HCMV has been detected in many tumor types [3–12], and the relationship between the virus and cancer has been investigated most extensively in glioblastoma multiforme (GBM) [13–16]. GBM is the most aggressive primary brain tumor with a median survival of 15 months and an unknown etiology [17]. GBM is characterized by the presence of a heterogeneous population of cells that are infiltrative, angiogenic and poorly metastatic [18]. Various reports demonstrated the presence of HCMV DNA, transcripts and/or proteins in GBM samples (reviewed in [16]). In addition, a number of studies showed that viral proteins can modulate the phenotype of GBM cells leading to cell proliferation [19], cell immortalization [20], cell migration [21, 22], stem cell maintenance [23, 24], angiogenesis [22, 25–27] and immunomodulation [28]. Although HCMV is not considered to be oncogenic, it is believed that viral proteins may increase cellular malignancy and contribute to tumor progression, a phenotype called oncomodulation [29]. In fact, studies suggest that extracellular factors released during HCMV infection can cause immunosuppression of the tumor microenvironment (reviewed in [30]), but the virus may also be more directly involved in the malignant phenotype of tumor cells (reviewed in [31]).
The standard GBM treatment is surgical resection followed by radiotherapy and chemotherapy. The cytotoxic treatment with Temozolomide (TMZ) and Carmustine (bis-chloroethylnitrosourea, BCNU) inserts alkyl adducts into the DNA backbone, causing errors during DNA replication due the formation of numerous DNA double-strand breaks (DSBs) not repaired by the cellular machinery. This lack in DNA repair ultimately leads to cell death [32]. Despite the standard treatment, less than 5% of GBM patients live longer than 5 years after diagnosis (reviewed in [33]), mainly because of the acquired and intrinsic drug resistance (reviewed in [34]). The main mechanism of chemotherapy resistance is mediated by O6-methylguanine DNA methyltransferase (MGMT), a suicide repair protein that removes DNA adducts formed at the O6-position of guanidine in response to alkylating agents, such as TMZ. MGMT expression is associated with poor patient response to alkylating therapies. Other molecular mechanisms and proteins involved in cell resistance to DNA damage are base excision repair (BER), ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (reviewed in [34]).

Studies demonstrated that HCMV upregulates the host BER machinery [35], activates the DNA damage response [36], and significantly reduces cyclobutane pyrimidine dimers in UV-exposed viral DNA [37], indicating a viral role in inducing DNA repair pathways that may limit DNA damage. On the other hand, HCMV was also found to cause DNA damage. For example, the HCMV protein UL76 was demonstrated to induce chromosomal aberrations [38], and the virus has been associated with specific damage at several loci in chromosome 1, 1q21, 1q23 and 1q42 [39, 40]. These findings lead to the speculation that the virus might either increase or decrease cell resistance to DNA damaging chemotherapeutic drugs.
To investigate these possibilities, we analyzed infection by low and high passage strains of HCMV in different GBM cell lines and their response to chemotherapy treatment in the presence and absence of the virus.
RESULTS

GBM cell lines are generally susceptible to HCMV infection but not equally permissive to viral replication

We and others have demonstrated that HCMV DNA and proteins are present in GBM tumors [14, 15]. However, few cells in the tumor bulk seem to contain viral DNA, transcripts and/or proteins [14], indicating that HCMV infection or replication is not equally efficient in all tumor cells. We therefore sought to investigate viral infection and replication using GBM cell lines and HCMV strains with different genetic backgrounds.

The GBM cell lines A172, TP365MG and U251MG were exposed to a low passage (TB40E) and a high passage (AD169) HCMV strain at MOI 3, and the presence of intracellular pp65 protein, a major component of HCMV particles, was evaluated at 2 h post infection (hpi) by confocal immunofluorescence microscopy. The pp65 associated with the input virus was detected in the vast majority (>90%) of cells in all infections, indicating no significant difference in virus entry among the different cell lines or HCMV strains (Fig. 1).

To examine the extent and temporal pattern of HCMV protein accumulation following infection, viral protein markers for the immediate-early (IE1-72kDa), early (pp52 also known as pUL44) and late (pp28) stages of viral replication were analyzed at different times post infection by immunofluorescence. Very different patterns of expression were observed in the three cell lines (Fig. 2 and 3). Controls are shown in Figures S1 and S2. The IE1-72kDa protein was detectable in only a small subset (<20%) of A172 cells at 16 hpi, and the proportion of cells staining positive for this protein further decreased over time (30‒96 hpi). Neither pp52 nor pp28 proteins were detected at any time point evaluated in this cell line. In contrast, IE1-72kDa was
observed in 40% to >70% of TP365MG and U251MG cells at 16 hpi, and up to almost 100% of these cells stained positive for IE1-72kDa at later times post infection. However, pp52 and pp28 protein accumulation differed markedly in the two cell lines. While most of the TP365MG cells had accumulated pp52 and pp28 by 96 hpi, the two proteins were detectable in only a small proportion of U251MG cells at this time.

Together the results show that all GBM cell lines tested are highly and equally susceptible to low and high passage strains of HCMV. However, the temporal pattern of immediate-early, early and late viral protein accumulation varies widely between the cell lines suggesting major differences in permissivity to HCMV replication determined by the cellular genetic background.

**HCMV replication reduces the number of viable GBM cells but does not increase tumor cell resistance to TMZ or BCNU**

Previous studies have shown that HCMV can augment or inhibit cellular DNA damage responses. Therefore, we aimed to evaluate the effect of HCMV infection on chemotherapy treatment of GBM cell lines with the DNA damaging drugs TMZ or BCNU.

Initially, viability of non-infected A172, TP365MG and U251MG cells in the presence of 25, 50 and 100 µM TMZ or BCNU for 96, 120 and 144 h was assessed by MTT assay. Compared to controls, both drugs caused a marked decrease of in the number of viable cells in a concentration-and time-dependent manner in all three cell lines (Fig. 4). However, the timing and extent by which cell viability was reduced varied with different combinations of drugs and cell lines. Further analyses were performed with drug concentrations that decrease cell viability by at least 30%, namely 50 µM
TMZ or BCNU for A172 and TP365MG cells and 75 µM TMZ or 35 µM BCNU for U251MG cells.

Subsequently, GBM cells were exposed to HCMV AD169 or TB40E at different MOIs (0.12, 0.6 and 3) or mock infection overnight and treated with TMZ, BCNU or solvent (DMSO or EtOH, respectively) for 96 h. Infection with either virus strain lead to a marked decline in the number of TP365MG and U251MG viable cells, even in the absence of drug. The reduction in the number of viable TP365MG cells was MOI-dependent for both AD169 and TB40E. However, only AD169 but not TB40E reduced the number of viable U251MG cells in an MOI-dependent fashion. As expected, TMZ and BCNU reduced cell viability in all cell lines in the absence of virus.

In U251MG cells, high MOI infection with AD169 and treatment with BCNU appeared to work additively in reducing the number of viable cells. Similar additive effects were less pronounced or absent upon treating AD169-infected U251MG cells with TMZ or infecting these cells with TB40E. Likewise, BCNU and TMZ did no exert significant additive effects when combined with AD169 or TB40E infection of TP365MG cells, most likely due to the pronounced reduction in the number of viable cells caused by HCMV alone in this system. Finally, there was no significant impact of HCMV on A172 cell viability, and no obvious additive effects between the viruses and drugs were observed in these cells (Fig. 5).

These results show that HCMV infection adversely affects viability of permissive GBM cells, but does not increase chemoresistance of the cell lines tested. Instead, HCMV may even increase susceptibility to BCNU under certain conditions.

**Intracellular presence of HCMV is required to reduce GBM cell viability**
It has been demonstrated that HCMV infection leads to the production and secretion of numerous cytokines some of which may adversely affect cell viability [41]. For example, the HCMV interleukin 10 homolog (cmvIL10) has been shown to mediate local tumor immunosuppression and activation of signal transducer and activator of transcription 3 (STAT3), a transcription factor associated with chemoresistance in ovarian cancer [42]. Our previous cell viability analyses were performed in de novo infected cells, suggesting that viral gene expression or replication caused the substantial reduction in the number of viable cells. However, this approach may mask possible indirect effects the virus may exert on the viability of uninfected cells by causing the release of soluble factors.

To test the possibility that soluble factors released from infected cells contribute to reduction in the number of viable cells even in the absence of infection A172, TP365MG and U251MG cells were inoculated with supernatants from MRC-5 cells infected with HCMV AD169 or TB40E for 48 h, and treated with the two chemotherapeutic drugs for 96 h. Expression of cmvIL10 in infected MRC-5 cells was confirmed by Western blotting (data not shown). Notably, only A172 cells treated with supernatants from AD169- or TB40E-infected cells and treated with TMZ exhibited a significant decrease in number of viable cells. This phenotype was not observed with any other combination of cells, viruses and drugs (Fig. 6).

These results confirm that reduced number of viable GBM cells caused by HCMV infection largely depends on the presence of the virus in the affected cells and likely results from viral gene expression and replication.

**HCMV infection reduces GBM cell proliferation and induces tumor cell death**
To further explore the impact of HCMV on GBM cell viability, we determined the amount of cell death in U251MG cells by fluorescence-activated cell sorting (FACS). U251MG cells were infected at MOI 3 overnight, and treated with low concentrations (25 µM) of TMZ or BCNU for 96 h. U251MG cells were chosen for this experiment due to the less pronounced decrease in cell viability upon viral infection compared to TP365MG cells, and the low drug concentration was used to limit cell debris during the analysis. IE1-72kDa was used as a marker for viral infection. Only a small proportion (<10%) of cells were positive for both propidium iodide (PI) and IE1-72kDa (Fig. 7a), suggesting that most of the infected cells were still viable. Further cell death analysis by trypan blue exclusion assay performed in TP365MG and U251MG cells confirmed a reduction in infected cell numbers when compared with controls. However, the percentage of viable cells in this assay proved to be higher than that obtained in the metabolic assays (Fig. 7b and Fig. 5).

These findings indicate that the reduced number of viable cells observed in HCMV-infected GBM cell lines is largely due to decreased cell proliferation, and that cell death makes a minor contribution to the overall effect.
Glioblastoma is a highly heterogeneous tumor, with genetic diversity existing both among patients and within individual tumors [43]. The intratumoral genetic heterogeneity can result in many phenotypes including populations of drug resistant subclones that are the main cause of treatment failure. HCMV is an infectious agent detected in GBM samples worldwide, although usually only in a subset of tumor cells [14]. There are also studies that failed to detect HCMV in GBM [44–46]. The causes underlying the presence or absence of HCMV in GBM cells and the consequences resulting from infection are not well understood. It has been demonstrated that HCMV can mediate DNA damage, but the virus can also induce DNA repair pathways [36, 37], phenotypes that could interfere in GBM chemotherapeutic treatment.

Our results demonstrate that three different GBM cell lines (A172, TP365MG and U251MG) are equally susceptible to HCMV infection. This conclusion is based on the intracellular presence of the major viral tegument component (pp65) in a similar number of cells at the beginning of infection. However, the permissivity of the cell lines to the temporal accumulation of HCMV proteins differs widely, again in a manner independent of the virus strain. While A172 cells undergo an infection that seems to be silenced or aborted at the immediate-early stage, TP365MG and U251MG cells are permissive to viral early and late gene expression. However, infection progresses more rapidly in TP365MG compared to U251MG cells. High passage (AD169) and low passage (TB40E) HCMV strains showed very similar phenotypes in terms of both infectivity and viral protein accumulation in the tested cell lines. These results suggest that the permissivity of GBM cells to HCMV replication is determined by cellular rather than viral factors. In fact, the three cell lines under investigation have different backgrounds with respect to important genes including CDKN2A, PTEN, p16, and p53.
Further studies are necessary to investigate the significance of mutations in these and other genes for permissivity to HCMV gene expression and replication in both cell lines and tumor tissue. Although we did not perform experiments in tumor tissue, the cell lines used in this work have been commonly used as experimental models in GBM research. Our results are compatible with the general view that HCMV is present in a limited number of cells in GBM tumor samples [14] implying that genetic differences between cells may result in different outcomes of infection.

Treatment of all three cell lines with TMZ or BCNU causes significant reduction in the number of viable cells in a dose-and time-dependent manner. In TP365MG cells both drugs caused a similar decrease in the number of viable cells. In contrast, U251MG cells treated with BCNU exhibited a decline in the number of viable cells of almost 100% at 144 h, a phenotype not observed with TMZ. It has been previously shown that BCNU induces cell death by necrosis and apoptosis in U138MG cells, a GBM cell line which carries mutant p53 and is therefore more susceptible to toxicity by BCNU than TMZ [51]. These findings are consistent with our own results, since U251MG cells carry mutant p53. However, the same phenotype is not observed in TP365MG cells which also have mutant p53, indicating that drug sensitivity is not exclusively linked to p53 status [52]. Notably, viral infection alone reduced the number of viable U251MG and TP365MG cells, which are both permissive to HCMV early and late gene expression, in a MOI-dependent manner. This phenotype was particularly pronounced in TP365MG cells, most likely due to their high levels of HCMV gene expression. No major effects were observed in A172 cells where viral protein accumulation is limited to immediate-early.

The concomitant presence of HCMV and TMZ or BCNU caused a further reduction in the number of viable cells. Interestingly, the reduction in the number of
viable TP365MG cells was MOI-dependent for both AD169 and TB40. However, only AD169 but not TB40E reduced the number of viable U251MG cells in an MOI-dependent fashion. This difference could reflect the genetic differences in the virus strains used in this study, such as the genes from the UL-b' region of the genome, which has been deleted from AD169 but are present in TB40E [53].

Overall, our results indicate that HCMV replication occurs differentially in GBM cell lines depending on their genetic background and that the adverse effects HCMV confers on GBM cells is likely caused mainly by reduction of cell proliferation, as shown by FACS and trypan exclusion assay, although cell death also contributes.

The effects HCMV may exert during chemotherapy treatment of GBM was previously investigated in several cell types including astrocytoma cells, breast cancer cells, neuroblastoma cells, peripheral blood lymphocytes and smooth muscle cells, but the results are controversial. HCMV-infected astrocytoma cells showed a decrease in apoptosis when treated with cisplatin, a phenotype also observed in etoposide-treated MDA-MB-231 breast cancer cells [54–57]. Likewise, UKF-NB-2A-AD169 cells, a neuroblastoma cell line persistently infected with HCMV, were more resistant to cisplatin-induced cell death [58]. Conversely, treatment with ganciclovir re-established sensitivity to chemotherapy in neuroblastoma and astrocytoma cell lines persistently infected with HCMV [56, 58]. HCMV has also been reported to increase resistance of glioma cancer stem cells to TMZ [59]. On the other hand, HCMV has been shown to increase genetic damage induced by bleomycin in peripheral blood lymphocytes [60, 61]. Interestingly, HCMV IE1-72kDa seems to stimulate while IE2-86kDa may suppress doxorubicin-induced apoptosis in smooth muscle cells [62]. We demonstrate here that HCMV does not increase resistance in three GBM cell lines. Instead, the virus causes a reduction in TP365MG and U251MG cell proliferation or survival both in the
absence and presence of the tested drugs. These findings highlight that HCMV not only exhibits tumor promoting potential but may also exert adverse oncomodulatory effects depending on tumor cell permissivity to viral replication. In tumor cells permissive to viral early and late gene expression, the virus may be oncostatic or oncolytic while the tumor promoting activities linked to viral immediate-early proteins may predominate in cells with restricted viral replication.

Many studies have been conducted to understand the relationship between HCMV and GBM, but we are still far from understanding the role the virus may have in tumor pathogenesis. Our results indicate that HCMV does not increase GBM cell resistance to TMZ and BCNU in vitro. However, further studies need to be done to examine whether the virus behaves similarly in other tumor cell lines and in in vivo.
**METHODS**

**Cell culture and virus production**

Human glioblastoma cell lines A172, TP365MG and U251MG were provided by Dr. Markus Riemenschneider, University of Regensburg. MRC-5 human embryonic lung fibroblast cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) at 37˚C in a humidified atmosphere with 5% CO₂.

HCMV BAD\textit{in}UL99GFP [63] was produced by electroporation, and AD169 and TB40E were produced by nucleofection of MRC-5 cells with the corresponding bacterial artificial chromosome clones (provided by Dr. Thomas Shenk, Princeton University). Cell supernatants containing virus were collected after complete cell lysis and cleared of cell debris by low-speed centrifugation. Cell-free virus stocks were stored at -80˚C. For virus titration, serial dilutions of stocks were plated on MRC-5 cells. At 24 hpi, cells were fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% BSA. Cells were labeled using a hybridoma supernatant of a primary mouse antibody to IE1 (1B12) [64] diluted 1:5, and a secondary goat anti-mouse antibody conjugated to Alexa Fluor 594 (Abcam, ab150116) diluted 1:1000. Nuclei were stained with Hoechst 33342 (Abcam, ab145597). Virus titers were expressed as plaque forming units per ml.

**Infection and viral protein detection**
To determine viral protein levels during a time course, A172, TP365MG and U251MG cells were plated in low serum (1%) media at a density of 100,000 cells on glass coverslips in 24-well plates and infected with HCMV AD169 or TB40E at MOI 3 overnight. After that, the inoculum was removed and replaced by fresh medium. Cells were maintained until 24, 48, 72, or 96 hpi (U251MG were also infected for 120 h) and subjected to standard or confocal epifluorescence microscopy. Briefly, cells were washed in PBS with 0.05% Tween 20 (PBS-T) and fixed with iced methanol for 15 min at -20°C. Methanol was then removed, coverslips dried, and cells blocked with 2% BSA in PBS-T for 1 h. Cells were incubated with primary antibodies mouse anti-pp65 (1:10), mouse anti-IE1-72kDa (1:200), mouse anti-pp52 (1:100) or mouse anti pp28 (1:10) (provided by Dr. Thomas Shenk, Princeton University) for 1 h, washed and incubated with a secondary goat anti-mouse antibody conjugated to Alexa 594 (1:1000) (Abcam, ab150116). Controls included mock infected cells with HCMV specific primary antibodies and secondary antibody, mock infected cells with secondary antibody only, HCMV infected cells with secondary antibody only (Fig. S1). In addition immunofluorescence experiments were performed in mock and HCMV infected cells using a mouse monoclonal antibody against the VP1 protein of JC polyomavirus (Abcam, ab34756) and the secondary anti-mouse conjugate from the above experiments (Fig. S2).

Coverslips were mounted with 4',6-diamidino-2-phenylindole (DAPI). For standard fluorescence microscopy, cells were observed using an EVOS FL Cell Imaging System (Thermo Fisher Scientific) and quantified with ImageJ software using the following formula: % infected cells = number of infected cells (red) / total number of cells (blue) × 100%. For confocal fluorescence microscopy, a series of z-images were recorded using a Leica TCS SP5 confocal microscope (Leica Microsystems).
Cell viability and cell death assays

TMZ (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide) and BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea) were purchased from Sigma-Aldrich, and stock solutions (100 mM) were prepared by dissolving the drugs in dimethyl sulfoxide (DMSO) or ethanol (EtOH), respectively. Solutions were aliquoted and stored at -20°C. For cytotoxicity analysis, A172, TP365MG and U251MG cells (10,000 cells/well) were incubated in triplicates overnight in 24-well plates. On the next day, the culture supernatants were replaced with fresh media containing 25, 50 or 100 μM TMZ or BCNU, and cells were incubated for 96, 120 and 144 h.

To determine the effects of HCMV during drug treatment, A172, TP365MG and U251MG cells were plated in medium with low serum (10,000 cells/well in 24-well plates), infected at MOI 3 overnight with AD169 or TB40E, and treated with chemotherapeutic drugs. A172 and TP365MG cell lines were treated with 50 μM BCNU or TMZ and U251MG cells were treated with 25 μM BCNU or 75 μM TMZ. In all cases, cells were incubated for 96 h and cell viability was analyzed by MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) or Alamar Blue assays (Thermo Fisher Scientific). The MTT assay was performed as previously reported[65, 66]. Briefly, after incubation with drugs the medium was removed, cells were washed twice with PBS, and 200 μL of a 0.5 mg/mL MTT solution in serum-free medium was added to each well. The cells were incubated for 4 h at 37°C, medium was removed, and formazan crystals were solubilized in 300 μl DMSO. The plate was shaken for 15 min and absorbance was measured at 570 nm. For the Alamar Blue assay, 100 μL reagent was added directly to the culture media and incubated for 3 h. Fluorescence was
measured at 530 nm excitation and 560 nm emission. In both assays results were compared to controls considered 100% viable, where cells were seeded and cultivated as described but not exposed to drugs and/or virus. DMSO or EtOH were used as solvent controls for TMZ and BCNU, respectively, at concentrations below 0.1% (concentration non-toxic to the cells). For trypan blue staining, A172, TP365MG and U251MG cells were plated and treated as in the cell viability assays. After treatment, cells were trypsinized, and the cell suspension mixed with 0.4% trypan blue solution at a 1:1 ratio. Live and dead cells were counted using a Neubauer chamber. The percentage of living cells was measured using the following formula: % live cells = number of live cells / (number of live cells + number of dead cells) × 100%.

To determine the effects of the HCMV secretome during drug treatment, MRC-5 cells were infected with AD169 or TB40E at low MOI for 48 h. Cell supernatants were centrifuged and filtrated through 0.4 µm filters. A172, TP365MG and U251MG cells were plated overnight (10,000 cells/well), inoculated with 200 µl supernatant overnight, and treated with chemotherapeutic drugs as described above. After 96 h, cell viability was analyzed by MTT assay.

**Flow cytometry**

U251MG cells (10,000 cells/well) were infected with HCMV BADinUL99GFP or mock-infected and treated with 25 µM TMZ or BCNU, incubated with PI for 15 min, trypsinized, and collected. After centrifugation and washing, cells were permeabilized with 0.1% Triton X-100, blocked with 3% BSA solution, and incubated overnight with anti-IE1 antibody (1:5) followed by goat anti-mouse Alexa 488 antibody (Abcam,
ab150113) (1:250) for 30 min. The cell pellet was re-suspended in PBS, and 10,000
events were analyzed in a BD FACSCalibur flow cytometer.

Statistical analysis

All experiments were performed at least twice (biological duplicates) in technical
triplicates. Statistical analysis was performed using two-way ANOVA (GraphPad
Prism). P<0.05 was considered statistically significant.

FINANTIAL SUPPORT

This study was supported by Fundação de Amparo a Pesquisa do Estado de
São Paulo, Brazil (FAPESP); Grant number: 2016/19925-2.

The authors declare that there is no conflict of interest.

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**FIGURE LEGENDS**

**Fig. 1. Detection of viral pp65 protein in HCMV-infected GBM cells.** A172, TP365MG and U251MG cells were infected with HCMV AD169 or TB40E at MOI 3 for 2 h. Confocal immunofluorescence microscopy was performed, and a series of z-images were recorded. The HCMV pp65 protein is shown in green, cell nuclei are shown in blue, and merge. Images were taken at magnification objective of 63x.

**Fig. 2. Expression of viral IE1-72kDa, pp52 and pp28 proteins in HCMV-infected GBM cells.** A172, TP365MG and U251MG cells were infected with HCMV AD169 or TB40E at MOI 3 for 16, 48 or 96 h and analyzed by immunofluorescence microscopy. HCMV proteins are shown in red, cell nuclei are shown in blue, and merge images is shown as well. Images were taken at magnification objective of 20x.
Fig. 3. Quantification of viral IE1-72kDa, pp52 and pp28 protein accumulation in HCMV-infected GBM cells. Immunofluorescence microscopy for HCMV immediately early (IE1-72kDa), early (pp52) and late (pp28) proteins was performed at different times post infection in A172, TP365MG and U251MG cells. Results are expressed as a percentage of infected cells divided by the number of total cells. At least 100 cells were counted for each immunofluorescence sample using ImageJ.

Fig. 4. Cell viability of GBM cells after treatment with TMZ and BCNU. The number of A172, TP365MG and U251MG viable cells was assessed by MTT assay after treatment with 25, 50 or 100 µM TMZ or BCNU for 96, 120 and 144 h. Data represent the mean (± standard deviation, SD) of three independent experiments done in triplicate, p<0.05.

Fig. 5. Cell viability of GBM cells after HCMV infection at different MOIs and treatment with TMZ and BCNU. Cell viability was assessed by MTT assay for A172 cells and by Alamar Blue assay for TP365MG and U251MG cells after overnight infection with HCMV AD169 or TB40E at the indicated MOIs and treatment with chemotherapeutic drugs. A172 and TP365MG cells were treated with 50 µM TMZ or BCNU for 96 h, and U251MG cells were treated with 75 µM TMZ and 35 µM BCNU for 96 h. Data represent the mean (± standard deviation, SD) of three independent experiments, * p<0.05, ** p<0.0036, *** p<0.0009; **** p<0.0001.

Fig. 6. Cell viability of GBM cells after incubation with supernatants from HCMV-infected cells and treatment with TMZ and BCNU. A172, TP365MG and U251MG cell viability was assessed by MTT assay after overnight incubation with supernatants
of AD169- or TB40E-infected MRC-5 cells for 48 h and treatment with chemotherapeutic drugs for 96 h. A172 and TP365MG cells were treated with 50 µM TMZ or BCNU, and U251MG cells were treated with 75 µM TMZ or 35 µM BCNU. Data represent the mean (± standard deviation, SD) of three independent experiments, ** p< 0.0098.

**Fig 7. Cell death analysis of HCMV-infected GBM cells.** (A) FACS analysis of U251MG cells infected overnight with BADinUL99GFP at MOI 3 (HCMV) or mock-infected after treatment with 25 µM TMZ or BCNU for 96 h. A monoclonal antibody against HCMV IE1-72kDa was used as a marker for infection and propidium iodide (PI) as a marker for cell death. (B) For trypan blue exclusion assay A172, TP365MG and U251MG cells were infected overnight at MOI 3 with HCMV AD169 or TB40E and treated with chemotherapeutic drugs for 96 h. A172 and TP365MG cells were treated with 50 µM TMZ or BCNU, and U251MG cells were treated with 75 µM TMZ or 35 µM BCNU. After treatment, cells were trypsinized and the cell suspension mixed with 0.4% trypan blue solution at a 1:1 ratio. Living and dead cells were counted using a Neubauer chamber. Data represent the mean (± standard deviation, SD) of three independent experiments, * p<0.05, ** p<0.0036, ***p<0.0009.

**Fig. S1. Control experiments for the analysis of viral protein expression.** Immunofluorescence microscopy in mock-infected cells with HCMV-specific primary antibodies for HCMV proteins and secondary antibody; mock-infected cells with secondary antibody only and HCMV-infected cells with secondary antibody only. HCMV proteins are shown in red, cell nuclei are shown in blue. Images were taken at magnification objective of 20x.
**Fig. S2. Control experiments for the analysis of HCMV protein expression.**

Immunofluorescence microscopy in mock-infected and HCMV infected cells with a mouse monoclonal antibody against the VP1 protein of JC polyomavirus and the secondary. IE1 staining in HCMV infected cells is shown as positive control. Positive control for HCMV infection. Viral proteins are shown in red, cell nuclei are shown in blue. Images were taken at magnification objective of 20x.
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**Fig. S1. Control experiments for the analysis of viral protein expression.**

Immunofluorescence microscopy in mock-infected cells with HCMV-specific primary antibodies for HCMV proteins and secondary antibody; mock-infected cells with secondary antibody only and HCMV-infected cells with secondary antibody only. HCMV proteins are shown in red, cell nuclei are shown in blue. Images were taken at magnification objective of 20x.
Fig. S2. Control experiments for the analysis of HCMV protein expression.

Immunofluorescence microscopy in mock-infected and HCMV infected cells with a mouse monoclonal antibody against the VP1 protein of JC polyomavirus and the secondary. IE1 staining in HCMV infected cells is shown as positive control. Positive control for HCMV infection. Viral proteins are shown in red, cell nuclei are shown in blue. Images were taken at magnification objective of 20x.