

Glioma-Associated Cytomegalovirus Mediates Subversion of the Monocyte Lineage to a Tumor Propagating Phenotype

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Abstract

Purpose: Cytomegalovirus (CMV) has been ubiquitously detected within high-grade gliomas, but its role in gliomagenesis has not been fully elicited.

Experimental Design: Glioblastoma multiforme (GBM) tumors were analyzed by flow cytometry to determine CMV antigen expression within various glioma-associated immune populations. The glioma cancer stem cell (gCSC) CMV interleukin (IL)-10 production was determined by ELISA. Human monocytes were stimulated with recombinant CMV IL-10 and levels of expression of p-STAT3, VEGF (vascular endothelial growth factor), TGF- β , viral IE1, and pp65 were determined by flow cytometry. The influence of CMV IL-10-treated monocytes on gCSC biology was ascertained by functional assays.

Results: CMV showed a tropism for macrophages (M Φ)/microglia and CD133+ gCSCs within GBMs. The gCSCs produce CMV IL-10, which induces human monocytes (the precursor to the central nervous system M Φ s/microglia) to assume an M2 immunosuppressive phenotype (as manifested by downmodulation of the major histocompatibility complex and costimulatory molecules) while upregulating immunoinhibitory B7-H1. CMV IL-10 also induces expression of viral IE1, a modulator of viral replication and transcription in the monocytes. Finally, the CMV IL-10-treated monocytes produced angiogenic VEGF, immunosuppressive TGF- β , and enhanced migration of gCSCs.

Conclusions: CMV triggers a feedforward mechanism of gliomagenesis by inducing tumor-supportive monocytes. *Clin Cancer Res*; 17(14); 4642–9. ©2011 AACR.

Introduction

In the United States, the prevalence of CMV in the adult population is 50% to 85% (1). As such, serum CMV positivity is common throughout the general population and CMV itself remains in an asymptomatic, latent state in healthy immune competent individuals. CMV has been shown to be present in glioma tumor cells but not in surrounding normal brain, or other neuropathologies (2–4). Malignant gliomas have been postulated to arise from transformed neural progenitor cells/glioma cancer stem cells (gCSC; ref. 5). These cells are permissive to CMV infection (6), resulting in abnormal differentiation (7) or inhibition of differentiation into normal astrocytes (8). The platelet-derived growth factor- α receptor

(PDGFR- α), expressed on neural progenitor/stem cells in the adult subventricular zone (9), is required for CMV infection as a result of binding the CMV envelope protein gB, resulting in phosphoinositide-3-kinase (PI3K) signaling (10). Blockade of PDGFR- α or its functional activity inhibits internalization and gene expression of CMV (10).

The etiological role of CMV in the genesis of gliomas is an area of significant ongoing controversy. Concurrent virology research has shown that CMV infectivity could predispose cells to oncogenesis. For example, US28, a chemokine receptor encoded by CMV that binds chemokines and activates various proliferation pathways, induces a proangiogenic and transformed phenotype in glioblastoma cells by upregulating vascular endothelial growth factor (VEGF). These US28-expressing cells promote tumorigenesis when injected into mice (11), probably mediated by the signal transducer and activator of transcription 3 (STAT3; ref. 12). In addition, the CMV immediate-early genes (UL123/122) that encode proteins crucial for initiating the viral replication cycle (13) have been investigated as a potential etiology for modulating an oncogenic phenotype in gliomas. However, this gene expression resulted in a variable response among various glioma lines—in some instances enhancing glioma cell proliferation while leading to growth arrest in others (14). CMV infection has also been shown to induce

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Translational Relevance

It is unclear whether the presence of cytomegalovirus (CMV) within malignant gliomas represents an epiphenomenon of tumor-mediated immunosuppression reactivating a latent infection or whether the virus is contributing to gliomagenesis. In this article, we describe a propensity of CMV infection for the glioma cancer stem cell (gCSC) and the macrophage (MΦs)/microglia populations. The gCSC production of CMV interleukin-10 subverts cells of the monocyte lineage, the precursor to the MΦs/microglia, to an immunosuppressive, tumor-supportive phenotype that has been shown to negatively influence outcome and treatment response. These data indicate that the natural mechanisms of CMV for evading the immunosystem are being appropriated in the glioma microenvironment to further potentiate tumor-mediated immune evasion and for the enhancement of gliomagenesis. In the future, anti-CMV therapeutics may be a novel approach for overcoming glioma-mediated immunosuppression.

phosphorylation of focal adhesion kinase, which can increase human malignant cell invasiveness (15), and the CMV IE2 protein has been shown to interact with histone deacetylase 2, resulting in increased transcriptional activity (16). Finally, CMV has been shown to alter microRNAs (17), which can play an important role in the development of cancers (18, 19). Cumulatively, these data suggest that the presence of CMV is not merely an epiphenomenon.

It is also unclear whether the tumor immunosuppressive environment supports susceptibility to infection with CMV or whether CMV induces tumor-mediated immunosuppression. As a mechanism to evade immune detection, the CMV antigen pp65 prevents the expression of other CMV antigens in the context of the MHC, inhibits natural killer (NK) cell toxicity, and degrades the HLA-DR α chain (20). In murine model systems, macrophages (MΦ) infected with murine CMV downregulate MHC class II molecules while transcriptionally inhibiting proinflammatory IFN- γ induction, essentially rendering these cells non-functional (21). CMV also encodes a consensus sequence of interleukin (IL)-10 (CMV IL-10; ref. 22) that can bind to the human IL-10 receptor and activate STAT3 (23, 24), a key molecular hub for tumorigenesis (25) and immunosuppression (26), especially in gliomas (27–30). CMV IL-10 has been found to inhibit both proliferation and the production of proinflammatory cytokines in monocytes (31), the myeloid precursors to MΦs and microglia (32).

The STAT3 pathway is active in gCSCs (33, 34), as determined by measurements of its activated form p-STAT3. In addition, it can be induced in diverse immune cells in the tumor microenvironment (35), which downregulates their antitumor immune responses. Specifically, p-STAT3 has been shown to suppress MΦ activation and to limit MΦ inflammatory responses (36), reduce NK cell and

neutrophil cellular cytotoxicity, and reduce the function of dendritic cells (35). Tumor-associated MΦs (TAM)/microglia become polarized via the STAT3 pathway toward the immunosuppressive and tumor-supportive phenotype (M2) that contributes to angiogenesis and tumor invasion (37). The role of STAT3 in mediating the immunosuppressive MΦs is further supported by studies in the murine model of endogenously arising heterogeneous gliomas that show a marked intratumoral influx of MΦs, a negative prognosticator for long-term survival (38).

By considering the parallel strategies of immune evasion seen in glioblastoma multiforme (GBM) and CMV infection coupled with the high frequency of identification of the virus in GBM tissue samples, we wanted to investigate whether viral activity directly contributes to the immunosuppressive environment. We therefore hypothesized that CMV infection within gCSCs potentiates tumor-mediated immunosuppression by inducing the STAT3 pathway and subverts the monocyte lineage to glioma-propagating phenotype.

Materials and Methods

Human tissues and PBMCs

This study was approved by the Institutional Review Board of The University of Texas M.D. Anderson Cancer Center (M.D. Anderson), Houston, TX, and conducted under protocol #LAB03-0687. Peripheral blood mononuclear cells (PBMCs; 30–50 mL) were isolated by centrifugation on a Ficoll-Hypaque density gradient (Sigma-Aldrich), washed with PBS, and treated with red cell lysis buffer as necessary. Tumors were washed in RPMI medium and dissected to remove blood products and surrounding non-tumor brain. Remaining bulk tumor was then enzymatically and mechanically digested using Tumor Cell Digestion Solution (Panomics) at 37°C and filtered through a 100 μ m membrane. The filtrate was pelleted by centrifugation and then resuspended in Percoll at a density of 1.03 followed by a 1.095 density underlay. Fluorescence-activated cell sorting (FACS) buffer (PBS + 2% BSA) was gently overlaid on the cell suspension, and the completed gradient was centrifuged for 15 minutes at 1,200 g with no brake. Cells were harvested from between the gradients, washed in FACS buffer (PBS + 2% BSA), and treated with red cell lysis buffer (Sigma-Aldrich). Cells were refiltered and washed as necessary. Cell viability was determined by using a ViCell 1.01 (Beckman Coulter).

Human glioma cancer stem cell derivation

The gCSC population has been previously described and characterized on the basis of the criterion of *in vivo* tumorigenic potential, pluripotent potential, limiting dilution assays, and cytogenetic characterization (34, 39–41). Supernatants from the gCSCs were collected and stored at –20°C for use as conditioned medium and ELISA analysis. The gCSCs were cultured *in vitro* with neurosphere medium consisting of DMEM (Dulbecco's modified Eagle's medium)/F-12 medium containing antibiotics, B27 growth

factor, and 20 ng/mL of both epidermal growth factor (EGF; Sigma-Aldrich) and fibroblast growth factor 2 (FGF-2; Sigma-Aldrich).

Human microglia/MΦs isolation and characterization

Microglia cells were purified using a Percoll (GE Healthcare) gradient (42) and phenotyped as previously described (43).

Derivation and isolation of purified CD14⁺ cells

PBMCs were prepared from healthy donor blood (Gulf Coast Blood Center) by centrifugation on a Ficoll-Hypaque density gradient (Sigma-Aldrich). Harvested PBMCs were purified with CD14 magnetic beads (MACS) and passed through a MS Column (MACS) to purify CD14⁺ monocytes. The percentage of CD14⁺ cells was determined to be approximately 95% by flow analysis. Purified monocytes were cultured in serum-free DC medium (Cell Genix). Recombinant CMV IL-10 was obtained from R&D Systems.

Cell lines and culture

Glioma cell lines U87, U251, and D54 were cultured in Dulbecco's DMEM F/12 media containing antibiotics and 10% FBS. The leukemia cell line HL-60 was cultured in RPMI medium supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 mmol/L Na pyruvate, 0.1 mmol/L nonessential amino acids, 10 mmol/L HEPES (pH 7.04), and 1× penicillin/streptomycin.

Surface and intracellular staining of cells

Ex vivo tumor cells and matched PBMCs in single-cell suspension were Fc blocked with human immunoglobulin (Ig) G (R&D Systems), except for the gCSCs used for US28 studies. Working concentrations of appropriate antibodies to surface markers were added [CD133 (MACS), CD45, CD11b, CD3, CD19, CD14 (BD Biosciences), CD163 (R&D), and US28 (Santa Cruz Biotechnology)] and incubated with the cells for 30 minutes in the dark at 4°C. A secondary antibody was required for US28 detection (Invitrogen). Matched isotype controls were included for each sample. Cells were pelleted by centrifugation and washed, followed by suspension in fix/perm buffer (eBioscience) for 2 hours in the dark at 4°C. Cells were rewashed with FACS buffer and 1× permeabilization buffer (eBioscience), and working concentrations of antibodies to intracellular proteins [IE1 (Millipore), pp65 (Pierce Biotechnology), gB (AbCam), and pSTAT3 (BD Biosciences)] were added to the appropriate wells. Cells were washed and resuspended in FACS buffer for data acquisition (FACSCaliber Becton Dickinson). Data were analyzed with Flow Jo Software (TreeStar).

ELISA

Supernatant medium obtained 24 hours after passage of the gCSCs was measured for CMV IL-10 (R&D Systems) as described. The CMV IL-10 capture antibody (Leinco Technologies) was used at 2.0 µg/mL with biotinylated detec-

tion antibody (R&D Systems) at 0.5 µg/mL. The lack of cross reactivity to human IL-10 was verified up to a concentration of 2 ng/mL.

Cytokine microarray

MACS-purified CD14⁺ monocytes were seeded at 1×10^6 on 24-well plates for 2 hours and then either left untreated or stimulated with human CMV IL-10 (10 ng/mL) for 48 hours at 37°C. After that, the supernatants were harvested, frozen at -80°C, and shipped to RayBiotech for L-507 antibody array and densitometry analyses.

Cell migration assay

The culture well inserts provided with a membrane pore size of 8 µm (BD Biosciences) were seeded with 200,000 gCSCs/insert and placed in 24-well culture plates containing either serum-free DC medium (Cell Genix; negative control), medium with 10% FBS (positive control), medium supplemented with CMV IL-10, or medium conditioned by monocytes cultured with or without CMV IL-10 for 24 hours. After overnight culture, all the cells that migrated through the membrane into the experimental medium were collected, pelleted with centrifugation, resuspended in a unit volume, and counted on the basis of Trypan blue exclusion.

Statistical analysis

The distribution of each continuous variable was summarized by its mean, SD, and range. The distribution of each categorical variable was summarized in terms of its frequencies and percentages. Continuous variables were compared between groups by a 2-sample *t* test if the data were normally distributed; otherwise a Wilcoxon rank sum test was used (44). Simple linear regression was used to assess the relationship between variables. *P* values less than 0.05 were considered to be statistically significant. Error bars represent SD.

Results

CMV expression within GBM-associated immune cells

To begin to investigate the relationship between CMV-infected gCSCs and intratumoral immune cells that may also harbor CMV, single-cell suspensions of newly diagnosed GBMs were analyzed ($n = 5$). CMV pp65 antigen expression was present in a subset of cells within these GBMs (Fig. 1A). Further analysis revealed distinct immune subpopulations that expressed pp65. Based on CD11b and CD45 expression, we found that both MΦs and microglia commonly expressed pp65; however, intratumoral T and B cells did not express pp65 (Fig. 1B). These data would indicate that CMV has a tropism for distinct immune cell populations within GBM.

CMV activity within gCSCs

Because gCSCs are rare in *ex vivo* glioblastoma tissue, we used a panel ($n = 4$) of well-characterized gCSCs (34, 40, 41) to further evaluate CMV activity. All gCSCs expressed

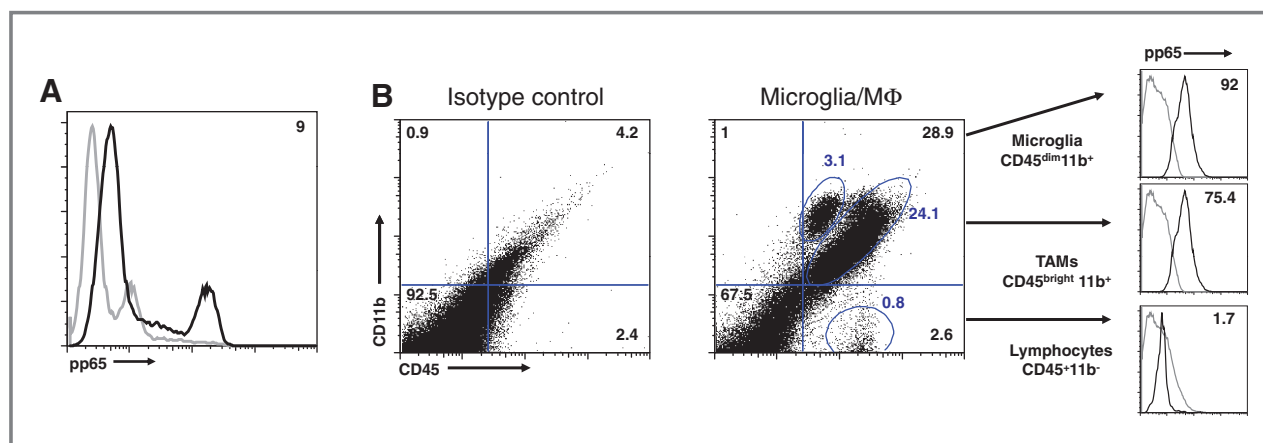


Figure 1. CMV antigen expression has a tropism for subsets of immune cells within GBMs. **A**, flow cytometry staining of GBM in single-cell suspension shows that only a subset of cells is positive for the CMV antigen pp65. The percentage of positive cells is denoted in the upper right quadrant of the histogram. **B**, flow cytometry gating strategy used to distinguish cells of monocyte lineage [such as microglia and MΦs (TAMs)] and lymphocytes based on CD11b and CD45 expression profile and intracellular staining of pp65. Each phenotype was separately analyzed for pp65 expression, as indicated by the histograms. The gray line represents the isotype control, and the black line shows the cells with positive staining for pp65. Percentages of cells are denoted in each quadrant and by their respective gates. The percentage of positive cells compared with the isotype control is shown in the right upper quadrant in the pp65 subanalysis panel. These data shown here are representative of the 5 newly diagnosed glioblastoma samples analyzed.

pp65, IE1, gB, and US28 (a representative example is shown in Fig. 2A). In addition, glioma cell lines such as U-87, U251, and D-54 express these CMV antigens (Supplementary Fig. S1). Further characterization of the CD133^{high} population compared with CD133^{low} population within the gCSCs ($n = 4$) showed higher levels of CMV IE1 in the CD133^{high} gCSC population relative to the CD133^{low} population (Fig. 2B). To assess whether the gCSCs are elaborating CMV IL-10, gCSC supernatants were tested by ELISA. All of the gCSCs ($n = 4$) produced CMV IL-10 at a range of 5.62 to 111.11 pg/mL/ 10^6 /day but no human IL-10.

CMV IL-10 induces CMV transcriptional activity in monocytes

There was a trend of elevated CMV pp65 expression levels in the PBMCs of the patients with newly diagnosed GBM who had a mean expression level of $33.6 \pm 25.2\%$ (range: 0–85; $n = 8$) in comparison with normal volunteers, who had a mean expression level of $17.5 \pm 8.4\%$ (range: 10.6–36.9; $n = 7$; Wilcoxon 2-sided test, $P = 0.27$; Fig. 3A). In a subanalysis of peripheral blood monocytes isolated from either GBM patients ($n = 3$) or normal donors ($n = 4$), precursors to glioma-associated MΦs and microglia, we found that the monocytes express the CMV antigen pp65 but relatively low IE1 (Fig. 3B). However, when these peripheral blood monocytes were treated with CMV IL-10, the expression of CMV IE1 in the monocytes was induced, suggesting an increase in transcriptional activity of the CMV within this subpopulation upon exposure to CMV IL-10.

CMV IL-10 subverts monocytes to an immune-suppressed phenotype

To ascertain whether CMV IL-10 can induce naive monocytes to an immunosuppressive phenotype, exogenous

CMV IL-10 was co-cultured with human CD14⁺ monocytes that were isolated from peripheral blood of normal donors ($n = 4$). In CD14⁺ monocytes exposed to CMV IL-10, there was a relative downregulation of MHC II and the costimulatory molecule CD86 but upregulation of the costimulatory inhibitory molecule B7-H1 (Fig. 4A). The exposure of the monocytes to CMV IL-10 also enhanced intracellular expression of p-STAT3, TGF-β1, and VEGF (Fig. 4B). To comprehensively analyze and potentially gain new insights into CMV IL-10-induced monocyte-elaborated cytokines, we used RayBiotech cytokine microarrays as a screen to evaluate candidates that could reciprocally enhance gCSC gliomagenesis (Supplementary Table S1). This CMV IL-10-induced immunosuppressive monocyte phenotype would be anticipated to enhance gliomagenesis.

CMV IL-10-exposed monocytes enhance gCSC migration

To functionally assess whether CMV IL-10 could induce the monocytes to secrete factors that would enhance gCSC migration, the gCSCs were assessed in standard migration assays upon exposure to supernatant culture medium from the CMV-IL10-treated monocytes. There was a 230-fold increase in gCSC migration relative to the control (CMV IL-10 containing medium alone) and a 40-fold increase in gCSC migration relative to exposure to supernatant medium from untreated monocytes (Fig. 5). Cytokine microarray analysis of the CMV IL-10-treated monocytes showed the upregulation of MIP-2, which has previously been shown to induce the mobilization of stem cells (45).

Discussion

Our data indicate that glioma-associated CMV may be contributing to gliomagenesis by appropriating the

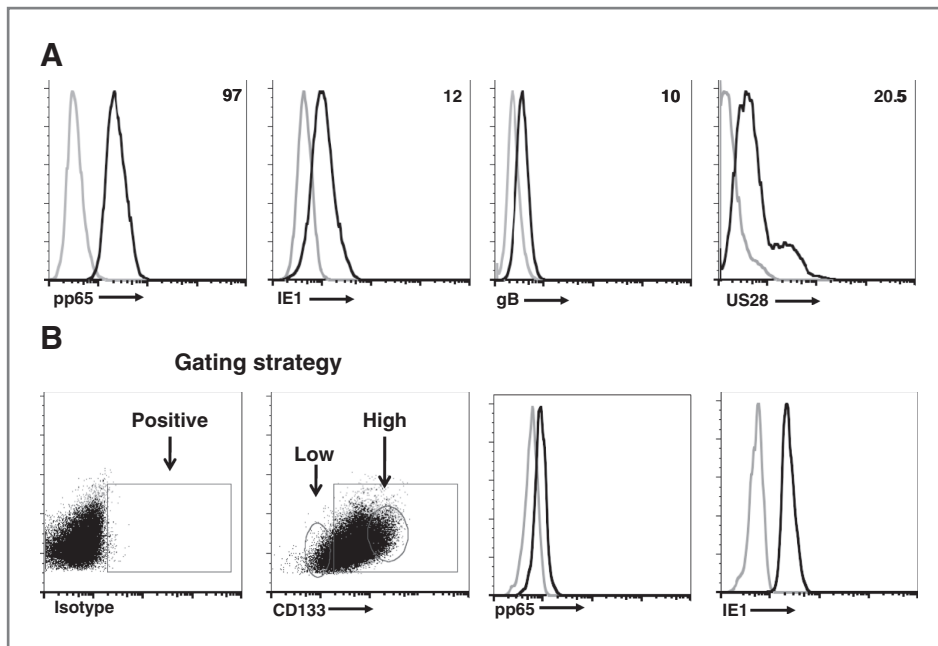


Figure 2. CMV antigen expression is enhanced in gCSCs. A, representative flow cytometry analysis of a gCSC sample stained for intracellular expression of CMV antigen expression of pp65, IE1, and gB as well as surface expression of US28. The percentage in the upper quadrant denotes positive cells relative to isotype. Similar data were obtained in 3 other gCSCs. B, flow cytometry gating strategy for gCSCs showing the rectangular gate-capturing cells staining positively for CD133 relative to the isotype control. The round gates capture the CD133^{low} and CD133^{high} phenotypes. The shift in histograms (solid black line) reflects increased CMV protein expression in the CD133^{high} population relative to the CD133^{low} (gray line).

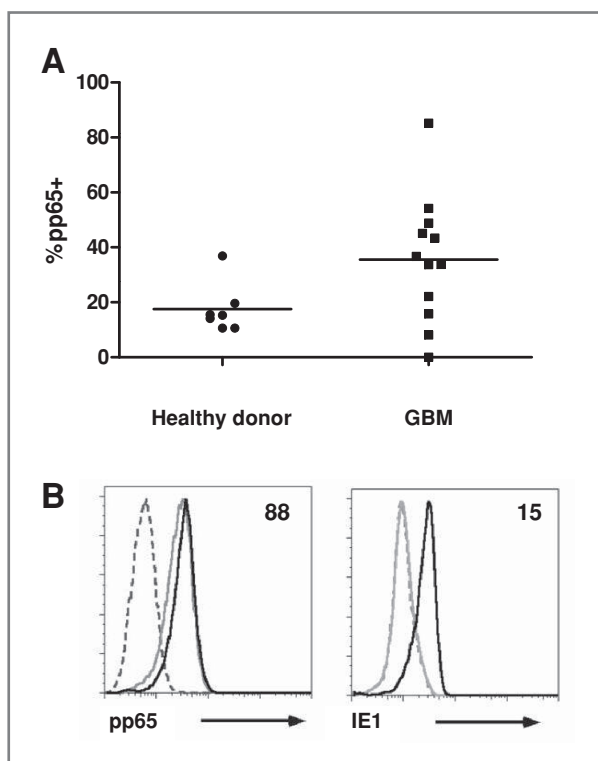
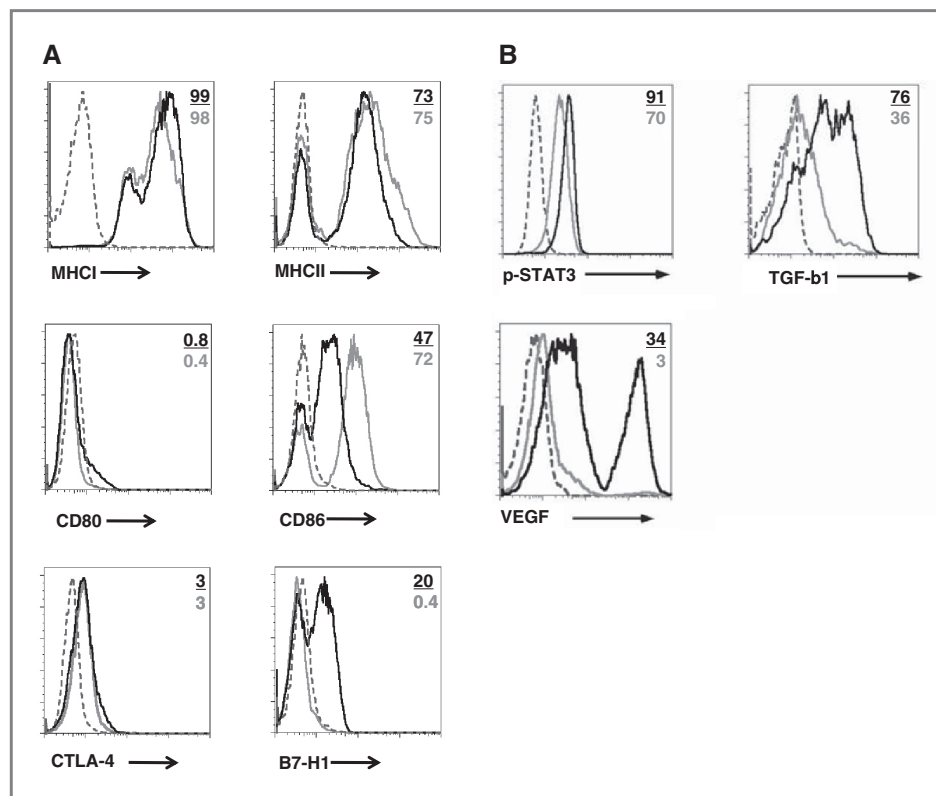


Figure 3. CMV IL-10 induces CMV transcriptional activity in monocytes. A, expression levels of pp65 in the PBMCs from GBM patients were found to be higher than in PBMCs from healthy donor controls although this was not statistically significant. B, purified CD14⁺ monocytes showed the expression of pp65 but relatively low levels of IE1. The dashed line represents isotype control and the gray line untreated monocyte staining. Upon exposure of the monocytes to CMV IL-10 as shown by the black line, there was upregulation of CMV IE1 but no further increase in pp65 expression.

monocytes/MΦs/microglia lineage to become tumor propagative/supportive. We found a tropism for CMV antigen expression, specifically pp65, in the gCSCs and MΦs/microglia. The role of CMV in contributing to glioma gliomagenesis through manipulation of the microenvironment is further supported by the finding of production of CMV IL-10 by the gCSCs and its subsequent effect on the MΦs/microglia precursor, the monocyte. We showed that CMV IL-10 activates IE1 in monocytes and that there is conversion to the immunosuppressive phenotype M2 that closely resembles the phenotype of TAMs and microglia (41, 42) that we have previously shown negatively influences outcome and treatment response in murine models of glioma (38). This subversion of the monocyte population by CMV IL-10 then enhances gliomagenesis by supporting angiogenesis (VEGF production), immunosuppression (TGF-β production), and invasiveness.

We are now proposing the following integrative model for the role of CMV in gliomagenesis. CMV, by binding to the PDGF receptor, gains access into the neural progenitor cells of the central nervous system (CNS; ref. 10). It is possible that CMV may integrate upstream of oncogenes or disrupt tumor suppressor pathways in the host genome as a mechanism of tumorigenesis, but to date, there has not been evidence to support this. Alternatively, the selective expression of US28, a chemokine receptor encoded by CMV (11), in the neural progenitor cell during the early phases of gliomagenesis would activate the STAT3. Indeed, we have shown that the expression of STAT3 in the neural progenitor cell correlates with malignant degeneration in murine glioma models (38). The CMV-infected gCSC then produces CMV IL-10 that triggers a feedforward mechanism that has potent immunosuppressive effects on monocytes (31).

Figure 4. CMV IL-10 induces an immunosuppressive phenotype in monocytes. A, in monocytes exposed to CMV IL-10 for 24 hours, there was downmodulation in the percentage of monocytes expressing MHC II and CD86 with upregulation of the costimulatory inhibitory molecule B7-H1. B, the exposure of the monocytes to CMV IL-10 caused an upregulation of intracellular p-STAT3, TGF- β 1, and VEGF. The dashed line represents the isotype control, the gray line shows monocytes cultured without CMV IL-10, and the black line shows monocytes cultured in medium containing CMV IL-10 at a dose of 10 ng/mL. Analysis was done after 48 hours in culture. This experiment was done with triplicate donor monocytes with similar results. The number in the right upper quadrant denotes the percentage of positive cells upon exposure to CMV IL-10 (black) and the percentage of positive cells without exposure to CMV IL-10 (gray).



The monocyte, the precursor to microglia and M Φ s (32), is recruited to the tumor microenvironment by the gCSCs through secretion of MIC-1, soluble colony-

stimulating factor, and TGF- β (41). Once in this environment, monocytes are exposed to the CMV IL-10 being produced by the gCSCs, and they then assume the immunosuppressive M2 phenotype. Consequently, latent CMV in the monocytes/M Φ s/microglia becomes active as reflected by IE1 expression. We suspect that the IE1 expression in the monocyte is not secondary to *de novo* infection of these cells (because they already express other CMV antigens) but rather that the CMV viral activity is increased by the CMV IL-10. Most importantly though is that the levels of STAT3, TGF- β , and VEGF are increased in the CMV IL-10-exposed monocyte lineage cells, further enhancing immunosuppression and further propagating gliomagenesis, including angiogenesis and the migration of the gCSC (Fig. 6).

Evidence is accumulating that CMV potentiates malignant progression of gliomas by having modulatory effects on the cell cycle, apoptosis, angiogenesis, cell invasion, and host immune response (46). Our findings that CMV IL-10 induces an immunosuppressive state are consistent with the findings of other investigators who have shown that CMV protein IE2 can upregulate TGF- β 1 gene expression (47, 48). Although the TGF- β 1 produced is a latent form, the authors postulate that in conditions with low pH and enzymatic digestion, it could be activated (47). The hypoxia, invasion, and inflammation associated with the glioblastoma microenvironment provide favorable conditions for activation of CMV-induced TGF- β 1. CMV has also been shown to further promote immunosuppression through

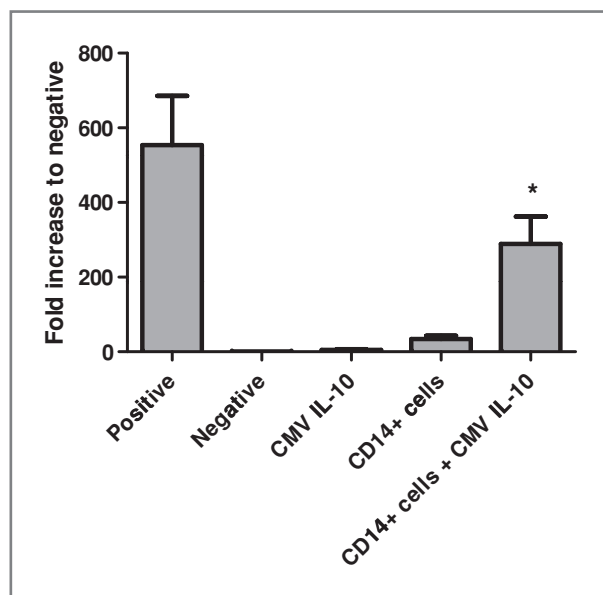


Figure 5. CMV IL-10-exposed monocytes enhance gCSC migration. The gCSCs were exposed to medium that was conditioned for 24 hours by monocytes treated with CMV IL-10 and appropriate controls. The CMV IL-10-treated monocyte supernatant increased gCSC migration in a chemotaxis assay compared with those grown in control medium. This experiment was done in duplicate. *, $P = 0.013$.

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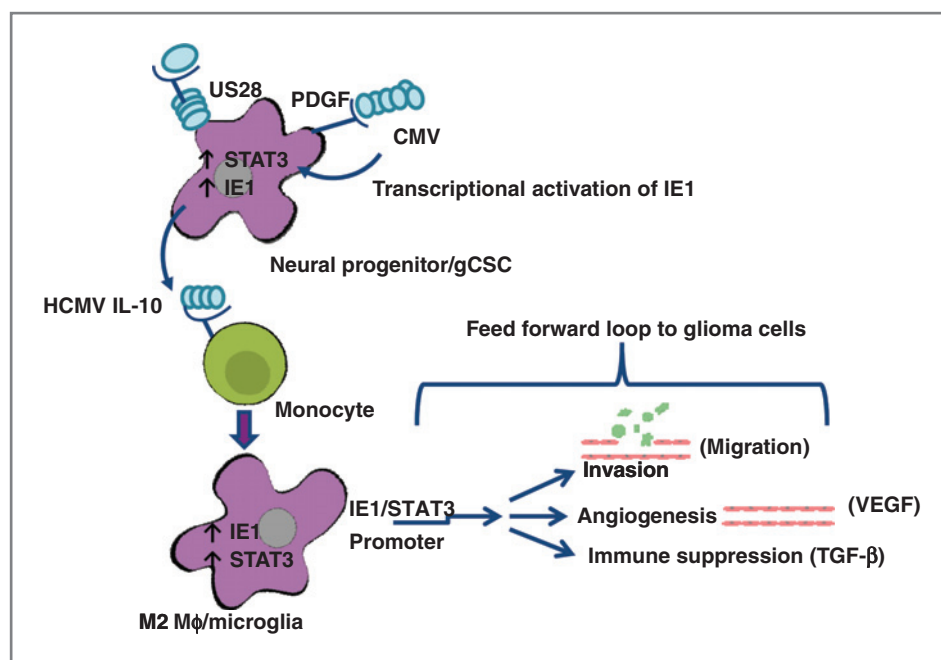


Figure 6. Schema showing the interrelationship between the gCSC and the monocyte. CMV infects the neural progenitor cell, the precursor to the gCSC, by surface attachment to the PDGF- α receptor. The CMV may possibly integrate into the host genome or interact with the STAT3 pathway to induce gliomagenesis. Viral activity, including the production of CMV IL-10, together with monocyte chemokines elaborated by the gCSC result in the preferential migration and conversion of the monocyte to an immunosuppressive and tumor-supportive M2 M ϕ /microglia (M2; TAM).

interference with processing of MHC class I molecules in the endoplasmic reticulum, which results in downregulation of expression (49). We suspect that the immunosuppressive M ϕ s further potentiate gliomagenesis in the later stages of tumor formation and may not necessarily be an inciting event since Walker and coworkers have shown that the influx of CNS M ϕ s is a late event in tumor formation (50). Collectively, the data argue against the idea that CMV merely plays a bystander role in glioblastoma pathology but is contributing to oncogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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