The Impact of Macrophage- and Microglia-Secreted TNFα on Oncolytic HSV-1 Therapy in the Glioblastoma Tumor Microenvironment

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Abstract

Purpose: Oncolytic herpes simplex viruses (oHSV) represent a promising therapy for glioblastoma (GBM), but their clinical success has been limited. Early innate immune responses to viral infection reduce oHSV replication, tumor destruction, and efficacy. Here, we characterized the antiviral effects of macrophages and microglia on viral therapy for GBM.

Experimental Design: Quantitative flow cytometry of mice with intracranial gliomas (±oHSV) was used to examine macrophage/microglia infiltration and activation. In vitro coculture assays of infected glioma cells with microglia/macrophages were used to test their impact on oHSV replication. Macrophages from TNFα-knockout mice and blocking antibodies were used to evaluate the biologic effects of TNFα on virus replication. TNFα blocking antibodies were used to evaluate the impact of TNFα on oHSV therapy in vivo.

Results: Flow-cytometry analysis revealed a 7.9-fold increase in macrophage infiltration after virus treatment. Tumor-infiltrating macrophages/microglia were polarized toward a M1, proinflammatory phenotype, and they expressed high levels of CD86, MHCII, and Ly6C. Macrophages/microglia produced substantial amounts of TNFα in response to infected glioma cells in vitro and in vivo. Using TNFα-blocking antibodies and macrophages derived from TNFα-knockout mice, we discovered that TNFα-induced apoptosis in infected tumor cells and inhibited virus replication. Finally, we demonstrated the transient blockage of TNFα from the tumor microenvironment with TNFα-blocking antibodies significantly enhanced virus replication and survival in GBM intracranial tumors.

Conclusions: The results of these studies suggest that FDA approved TNFα inhibitors may significantly improve the efficacy of oncolytic virus therapy. Clin Cancer Res; 1–12. ©2015 AACR.

Introduction

Glioblastoma (GBM) is one of the most common and deadly types of primary brain tumors. These tumors are characterized by widespread invasion, extensive angiogenesis, and resistance to cell death (1). These features along with a restrictive blood–brain barrier severely limit treatment options and result in a median patient survival of 15 months (2).

Oncolytic herpes simplex viruses (oHSV) are viruses genetically modified to specifically infect, replicate in, and target cancer cells for destruction. oHSVs represent a promising treatment modality for patients with GBM, and in clinical trials these viruses are safe and well tolerated (3). Early-phase clinical trials have produced promising results, and there is currently a phase III clinical trial for patients with advanced melanoma (NCT00769704; refs. 4–6). The success of oHSV-derived therapeutics is thought to depend on the oncolytic destruction of tumor cells and the activation of antitumor immune responses that can potentially lead to long-term cancer remission. However, the proinflammatory immune responses generated by viral infection can also antagonize oHSV replication and spread. Innate immune responses destroy replicating virus and reduce tumor cell killing, and several studies have demonstrated the negative effects of innate immune responses to oHSV treatment (7–9).

Microglia and infiltrating macrophages are thought to be significant mediators of the innate immune response to viral infection in the CNS (10–14). Depletion of these cells with clodronate liposomes or cyclophosphamide (CPA) reduces antiviral responses and improves oHSV efficacy (15–20). As a result of these preclinical studies, the combination of oncolytic measles virus with CPA is currently being evaluated in a phase I clinical trial for multiple myeloma (ClinicalTrials.gov Identifier: NCT00450814). Although these studies highlight the importance of modulating early immune responses to oHSV infection, the depletion of all phagocytic cells with...
Translational Relevance

Glioblastoma is one of the most common and deadly types of primary brain tumors, and patients diagnosed with these tumors have a median survival of only 15 months. Oncolytic herpes simplex viruses (oHSV) represent a promising therapy for glioblastoma, and these viruses are currently being tested in patients for safety and efficacy. Innate immune responses to viral infection are thought to reduce oHSV replication, tumor destruction, and efficacy. In this study, we investigated the antiviral functions of microglia and macrophages in oHSV therapy for glioblastoma. We demonstrated that the inhibition of TNFα could significantly enhance virus replication and efficacy in vivo. The results of these studies suggest that FDA approved TNFα inhibitors may significantly enhance patient outcomes in oHSV clinical trials.

Materials and Methods

Cell lines

Vero, LN229, U87ΔEGFR, U251-T2, and U251-T3-mCherry cells were maintained in DMEM supplemented with 10% FBS. U251-T2 and U251-T3-mCherry cells were created in our laboratory (May 2009) as tumorigenic clones of U251 cells by serially passaging these cells two and three times in mice, respectively. Monkey kidney epithelial-derived Vero cells and U87ΔEGFR cells were obtained in April 2005 from Dr. E. Antonio Chiocca (Ohio State University, Columbus, OH). LN229 cells were obtained in January 2005 from Erwin Van Meir (Emory University, Atlanta, Georgia). GB30 neurospheres were maintained as tumor spheres in Neurobasal Medium supplemented with 2% B27, human EGF (50 ng/mL), and basic fibroblast growth factor (50 ng/mL) in low-attachment cell culture flasks as previously described (21).

Western blots

Protein expression was determined using antibodies to detect the indicated proteins. Equal amounts of protein were loaded in each lane using a Biorad protein assay. Membranes were incubated with the appropriate antibodies: CD45, CD11b, MHCII, CD86, CD16/CD32 antibody (eBioscience). The blots were developed with peroxidase-conjugated secondary antibodies and visualized using the ECL Western blotting detection reagents (Amersham). The exposed films were scanned, and density was measured using NIH ImageJ software.

Microglia and macrophage antibody staining

Staining of surface antigens were performed as previously described (22). Briefly, Fc receptors were blocked with anti-CD16/CD32 antibody (eBioscience). Cells were then incubated with the appropriate antibodies: CD45, CD11b, MHCII, CD86, LY6C, LY6G, and CD16 (eBioscience) for 45 minutes. Cells were resuspended in FACS buffer (2% FBS in HBSS with 1 mg/mL sodium azide) for analysis. Non-specific binding was assessed via isotype-matched antibodies. Antigen expression was determined using a Becton-Dickinson FACS Caliber four color cytometer. Ten thousand events were recorded for each sample and

clodronate liposomes or total immune suppression with high doses of CPA does not specifically address the mechanism by which macrophages and microglia limit oHSV replication, spread, and efficacy.

In this study, we investigated the impact of microglia and macrophages in oHSV therapy for GBM. Quantitative flow-cytometry analysis of mice with intracranial gliomas treated with oHSV revealed significant changes in the activation and infiltration of macrophages and microglia in oHSV-treated animals relative to untreated mice. To evaluate the impact of these cells on oHSV propagation, we developed an in vitro coculture system with infected glioma cells and microglia/macrophages. In these studies, macrophages and microglia significantly reduced virus replication. Furthermore, we identified microglia/macrophage-secreted TNFα as a major factor that reduces viral replication through the induction of apoptosis in infected cells. In coculture assays, we were able to rescue changes in virus replication with TNFα-knockout macrophages or TNFα function-blocking antibodies. Finally, we demonstrated that the specific inhibition of TNFα produced by the tumor microenvironment could significantly enhance virus replication and efficacy in vivo. The results of these studies suggest that FDA approved TNFα inhibitors may significantly enhance patient responses in oHSV clinical trials.
isotype-matched conjugate. Data were analyzed using FlowJo software (Tree Star).

Image acquisition
Fluorescent and bright field images were acquired using an Olympus IX81 epi-fluorescence microscope equipped with a QImaging Retiga 2000R FAST camera and Olympus objectives. Image-Pro software (Version 6.2) was used for image acquisition. For luciferase imaging, mice received an i.p. injection of luciferin (Caliper Life Sciences) and the luciferase signal was visualized/quantified using an IVIS Lumina II imaging system.

Animal surgery
All animal experiments were performed in accordance with the Subcommittee on Research Animal Care of The Ohio State University guidelines and were approved by the Institutional Review Board. Six- to 8-week-old female athymic nude mice (NCI) were used for in vivo tumor studies. Intracranial surgeries were performed as previously described with stereotactic implantation of 100,000 U87ΔEGFR (22). Tumors were treated with HBSS, rQNestin34.5, or rHSVQ1-Luciferase virus at the location of tumor implantation. For antibody studies, mice were treated via i.p. injection at the days indicated with 400 μg of anti-murine TNFα antibody (XT3.11) or isotype control antibody (BE0094; BE0088) from BioXCell. Animals were euthanized when they showed signs of morbidity.

Statistical analysis
The Student t test or one-way ANOVA with Bonferroni multiple comparison post hoc tests were used to analyze changes in cell killing, viral plaque forming assays, luciferase imaging experiments, and flow-cytometry assays. In survival assays, Kaplan–Meier curves were plotted and the log-rank test was used to determine statistical significance. All statistical analyses were performed with the use of GraphPad Prism software (version 5.01). A P value of <0.05 was considered statistically significant. Derived P values are identified as *, P < 0.05; **, P < 0.01; *** P < 0.001.

See Supplementary Materials and Methods.

Results
oHSV Therapy activates microglia in vivo
In GBM animal models, microglia comprise 13% to 34% of all viable cells in the tumor (25). Similar ranges are seen in human tumors, and these observations underscore the importance of this cell type in the context of oHSV therapy (26, 27). The ability of microglia in the tumor microenvironment to switch from a glioma-supportive role to an antiviral state following oHSV treatment has not been well studied. To examine changes in microglia activation following oHSV infection in vivo, we treated mice with established U87ΔEGFR intracranial tumors with oHSV or PBS (injection control). These mice were euthanized 3 days following treatment, and we analyzed the tumor- and nontumor-bearing hemispheres for microglia (CD11b+CD45+), MHCII expression (Fig. 1A). We observed an 8.75-fold increase in microglia MHCII expression in oHSV-treated mice compared with PBS-treated animals (P < 0.001; Fig. 1B). Interestingly, oHSV therapy upregulated mean microglia MHCII expression in both the tumor- and nontumor-bearing hemispheres of the brain, but this increase was higher in the tumor bearing hemisphere (16.33% MHCII+) compared with the nontumor-bearing hemisphere (8.23% MHCII+; Fig. 1C).

oHSV therapy increases macrophage infiltration into the brain tumor microenvironment
Microglia activation induces the expression of various cytokines and chemokines that can stimulate the migration of immune cells into the CNS (14). Macrophages are important mediators of this innate immune response to viral infection, but the extent of macrophage infiltration into the CNS following oHSV therapy is unknown. To quantify the impact of oHSV-induced macrophage migration, we treated mice with established intracranial U87ΔEGFR tumors with oHSV or PBS as described earlier. We observed a 7.96- and 5.70-fold increase in macrophage (CD11b+CD45+) infiltration into the tumor- and nontumor-bearing hemispheres following oHSV infection, respectively (n = 5/group; P < 0.001 and P < 0.05; Fig. 1D and E). Although oHSV therapy strongly induced macrophage infiltration into both hemispheres, this increase was significantly higher in the tumor-bearing hemisphere (P < 0.001; Fig. 1D and E). Although macrophages comprised the bulk of the innate immune cell infiltrate, other innate immune populations are known to migrate into the CNS following viral infection (7, 28). We examined the percoll isolated cell populations for Ly6G+ neutrophils and CD160+ natural killer (NK) cells, and we found few NK cells or neutrophils in the CD11b+CD45+ populations at this time point (Supplementary Fig. S1).

oHSV therapy increases macrophage activation in the brain tumor microenvironment
oHSV therapy induced significant macrophage infiltration into the brain tumor microenvironment, but the phenotype and activation of these cells remained unknown. Depending on their polarization, macrophages can promote an immunosuppressive or proinflammatory tumor microenvironment. The activation status of these infiltrating cells is crucial to understanding how these cells contribute to oHSV therapy for GBM. To determine the polarization status of infiltrating macrophages, we evaluated the expression of the classic activation markers CD86, Ly6C, and MHCII. We observed significant increases in the percentages and cell numbers of macrophages (CD11b+CD45+) positive for CD86+ and Ly6C+ following oHSV treatment compared with PBS treatment (P < 0.001; P < 0.001, respectively; Fig. 2A and B). Although the percentages of MHCII-positive macrophages (CD11b+CD45+) in the tumor environment between oHSV and control treatments did not change, we observed a 9-fold increase in the total numbers of MHCII-positive macrophages infiltrating the tumor-bearing hemisphere following oHSV therapy compared with control-treated mice (P < 0.001; Fig. 2C). The surface expression of these three activation markers increased on macrophages(CD11b+CD45+) in both the treated and untreated hemispheres, but the treated hemispheres contained higher percentages of macrophages that expressed CD86 and Ly6C (Fig. 2A–C). Together, these data suggested that the infiltrating macrophages were polarized toward a proinflammatory state.
Coculture of oHSV-infected tumor cells with microglia or macrophages reduces viral replication in vitro

oHSV treatment significantly activated microglia/macrophages in vivo, but the effects of these polarized immune cells on virus replication remained unknown. To determine the functional consequences of this microglia/macrophage activation, we developed an in vitro coculture system. Human glioma cells were infected with oHSV at an MOI of 2, washed to remove unbound virus, and then overlaid with murine macrophages (RAW264.7) or microglia (BV2; Fig. 3A). Figure 3A is representative of co-cultures done with murine macrophages (RAW264.7) or microglia (BV2). To specifically examine the microglia/macrophage response toward infected cells and not toward free virus, the infected cells were cultured for less than 12 hours to prevent the lytic burst of tumor cells and the infection of microglia/macrophages. Compared with infected glioma cells alone, culturing infected cells with microglia or macrophages reduced viral titers by 37.28% and 69.99%, respectively (P < 0.01; P < 0.001; Fig. 3B and C). This decrease in virus replication was also accompanied by significant phenotypic changes 12 hours after infection. Uninfected glioma cells were adherent with extensive filopodia. Following infection, these cells became rounded, but remained adherent (Fig. 3D). Similarly, uninfected glioma cultured with microglia/macrophages revealed no significant changes in morphology. Interestingly, when infected glioma cells were cultured with macrophages or microglia, the microglia/macrophages surrounded the infected tumor cells and formed tight rosette-like clusters that became nonadherent (Fig. 3D; Supplementary Fig. S2A and S2B).

Macrophage and microglia secreted TNFα inhibits virus replication

Culturing infected glioma cells with microglia or macrophages significantly decreased virus replication, but how these cells reduced virus propagation remained to be elucidated. TNFα is a pleiotropic cytokine whose expression is significantly upregulated in response to viral CNS infections (14, 29). To test whether TNFα produced by activated macrophages/microglia could limit viral replication in glioma cells, we determined the levels of TNFα secreted by microglia and macrophages in our coculture system using a species-specific ELISA (Supplementary Fig. S3). Using a murine-specific TNFα ELISA, we observed that microglia and macrophages produced
significant amounts of TNFα in response to infected tumor cells. Compared with uninfected cocultures, oHSV infection increased macrophage- and microglia-secreted TNFα by 35.42- and 9.00-fold, respectively (P < 0.001; P < 0.01; Fig. 4A and B). Interestingly, we observed a 57.11% and 33.66% decrease in macrophage- and microglia-secreted TNFα when these cells were cultured with uninfected tumor cells compared with being cultured alone, respectively (Fig. 4A and B). In support of these in vitro data, we also observed a significant increase in murine-secreted TNFα in the brain and serum following oHSV treatment in intracranial xenografts (P < 0.001; P < 0.01, respectively; Supplementary Fig. S4A and S4B). This result is concordant with previously published work demonstrating that macrophages and microglia produce large amounts of TNFα in response to HSV infection in the CNS (29). Next, we determined whether the levels of TNFα produced in these cocultures were sufficient to reduce virus replication in glioma cells. Treatment of infected cells with 1,000 or 2,000 pg/mL of recombinant human TNFα resulted in 34.29% and 40.73% reductions in virus replication, respectively (P < 0.001; Fig. 4C). Similar results were obtained when infected glioma cells were treated with recombinant murine TNFα (Supplementary Fig. S5A). Visual inspection of infected cells treated with soluble TNFα also revealed surprising morphologic changes. oHSV-infected glioma cells treated with TNFα became rounded and nonadherent. The cells resembled infected cultures with microglia/macrophages (Fig. 4D, Fig. 3D and Supplementary Fig. S5B). We did not observe any morphologic changes or reductions in cell viability in multiple uninfected glioma cell lines treated with TNFα (Supplementary Fig. S6A and S6B). Additional experiments with uninfected glioma cells treated with varying doses of TNFα for 60 hours also did not reduce cell proliferation (P < 0.001 for all doses; Supplementary Fig. S6C).

Secreted TNFα induces apoptosis in oHSV-infected cells

Macrophage- and microglia-secreted TNFα significantly reduced virus replication in oHSV-infected cells, but the mechanism of TNFα-directed virus inhibition remained to be determined. High-magnification images of oHSV-infected cells treated with TNFα revealed significant changes in cell morphology. Unlike oHSV-infected cells alone, the addition of TNFα resulted in significant membrane blebbing (white arrows), cell shrinkage, and a loss of adherence, all features characteristic of cells undergoing apoptosis (Fig. 4D). On the basis of these observations, we hypothesized that TNFα-induced apoptosis in infected cells resulting
in reduced virus titers. To investigate whether TNFα was inducing apoptosis in infected cells, we conducted immunoblot assays for caspase-8, cleaved caspase-3, and cleaved PARP. We observed significant caspase-8, caspase-3, and PARP activation in cells treated with oHSV and TNFα. We did not observe significant activation of these proteins in glioma cells treated with TNFα or oHSV alone (Fig. 4E; Supplementary Fig. S7).

Inhibition of macrophage- or microglia-secreted TNFα increases oHSV replication in vitro

Because macrophage- and microglia-secreted TNFα reduced virus replication by inducing apoptosis in infected cells, we hypothesized that the inhibition of macrophage/microglia–produced TNFα would significantly improve virus replication. To determine whether inhibiting macrophage TNFα was sufficient to rescue virus replication, we conducted coculture assays with freshly isolated wild-type or TNFα-knockout (TNFα−/−) bone marrow–derived macrophages (BMDM). Phase-contrast microscopy of these cultures revealed significant morphologic differences between the two groups. Consistent with our previous results, a majority of the infected glioma cells cultured with wild-type BMDMs formed nonadherent clusters and exhibited significant membrane blebbing indicative of apoptosis. In contrast with these observations, infected glioma cells cultured with TNFα−/−BMDMs were adherent and showed substantially less membrane blebbing (Fig. 5A). These observations correlated with changes in virus titers; culturing infected glioma cells with TNFα−/−BMDMs significantly rescued virus replication compared wild-type BMDMs (P < 0.05; Fig. 5B). In a similar experiment, we found the addition of murine-specific TNFα-blocking antibodies rescued the reduction in virus replication in infected glioma cells when cultured with BV2 microglia (P < 0.05; Fig. 5C and D).

**Figure 3.** Microglia and macrophages reduce virus replication in tumor cells in vitro. **A,** schematic of microglia/macrophage and tumor cell cocultures. Tumor cells (oval-shaped cells) were infected with oHSV (HSVQ1; black dots) at an MOI of 2. Unbound virus was washed away and microglia or macrophages (branched cells) were overlaid on the infected glioma cells. The cells were cultured for 12 hours and the viral titers were determined by a standard plaque formation assay. **B** and **C,** viral titers of glioma cells infected alone or cultured with BV2 microglia (n = 4/group; B) or RAW264.7 macrophages (n = 4/group; C). Data shown are mean virus titer ± SD. **D,** representative images of glioma cells cultured with microglia and macrophages with and without oHSV infection 12 hours after infection (n = 4/group). **,** P < 0.01; ***,** P < 0.001.
the translational relevance of these results for oHSV therapy, we tested whether TNFα blockade could enhance virus replication in vivo. In these experiments, athymic nude mice were implanted subcutaneously with U87ΔEGFR human GBM tumors. When the tumors reached an average volume of 143 mm³, the mice were treated with a single dose of oncolytic virus. Mice were also administered a murine-specific TNFα-blocking antibody or a control antibody. Mice were given antibody 1 day before virus injection, the day of virus administration, and on days 1, 2, and 3 after virus treatment. In these studies, a luciferase-expressing oHSV was used to visualize virus replication. We observed a significant enhancement in virus propagation in vivo (as measured by luciferase encoded by virus) in mice treated with a TNFα-blocking antibody as compared with a control IgG antibody on days 1, 2, and 3 following oHSV administration (n = 5/group; P < 0.02; P < 0.01; P < 0.02, respectively; Fig. 6A and B). These results suggested that the inhibition of TNFα produced by macrophages and the tumor microenvironment was sufficient to increase virus replication in vivo.

Finally, we conducted intracranial GBM studies to determine whether TNFα blockade could enhance the survival of mice treated with oHSV. In these studies, mice were implanted intracranially with U87ΔEGFR human GBM cells and treated with oHSV 8 days later (2 x 10⁵ pfu rHSVQ1-luc). The antibody dosing regimen from the subcutaneous tumor experiments was used in this study. Mice treated with oHSV and a murine-specific TNFα-blocking antibody lived significantly longer than those treated with oHSV and an isotype control antibody (P = 0.026), TNFα-blocking antibody alone (P = 0.0003), or with an isotype control antibody alone (P = 0.0003; Fig. 6C). These results...
suggested that the combination of TNFα-blocking antibodies may enhance oHSV therapeutic efficacy for GBM.

Discussion

oHSV therapy is a promising treatment modality for GBM. The success of oHSV-derived therapeutics depends on both the oncolytic destruction of tumor cells and the activation of long-term, antitumor immune responses. Although the innate immune response is important for activating adaptive responses, the innate responses to oHSV therapy can also inhibit virus replication and oncolytic tumor cell killing. Depletion of macrophages and microglia with clodronate liposomes and CPA has previously been shown to reduce antiviral responses and improve oncolytic virus efficacy for GBM (9, 15–20, 30–32). The combination of oncolytic measles virus with CPA is currently being evaluated in a phase I clinical trial for multiple myeloma (ClinicalTrials.gov Identifier: NCT00450814). Recently, NK cells were shown to help coordinate the innate immune response to oHSV therapy, and the depletion of these cells was found to enhance oncolytic virus (OV) efficacy for GBM (7). Neutrophils have also been shown to limit OV dissemination in part through the release of neutrophil extracellular traps (33). Collectively, these studies suggest modulating early innate immune responses to achieve the optimal balance between viral replication and inflammation is critical to the clinical success of oHSV therapies.

Although microglia and infiltrating macrophages are thought to be the primary mediators of the innate immune response to oHSV infection for GBM, the mechanism by which these cells limit virus replication and therapeutic efficacy has not been well studied (16). Here, we quantified the extent of microglia/macrophage activation and infiltration following oHSV treatment. Although microglia are the resident immune cells of the CNS, in this study, we observed infiltrating macrophages outnumbered microglia more than 2:1 in the tumor microenvironment following oHSV infection. These results suggested that monocyte-derived macrophages may be the dominant cell type that controls oHSV infection.

Figure 5.
Inhibition of microglia/macrophage–secreted TNFα increases virus (rHSVQ1) replication in vitro. A, representative images of U251-T2 glioma cells infected at an MOI of 2 cultured with bone marrow–derived macrophages derived from wild-type or TNFα knockout mice for 12 hours. Large representative images are taken at a ×4 magnification with the insets taken at a ×20 magnification (white arrows indicate blebbing). B, 12 hour viral titers of cultures described in A. Data shown are mean virus titer ± SD. C, schematic of experimental setup using murine specific TNFα antibodies to block microglia (branched cells) secreted TNFα in cocultures with infected glioma cells (oval-shaped cells). D, quantification of virus titer obtained from infected glioma cells cultured with BV2 microglia with IgG or anti-murine TNFα–blocking antibody (1,800 ng/mL). Data shown are mean virus titer ± SD. **, P < 0.01; *, P < 0.05.
Although infiltrating macrophages primarily increased in the tumor-bearing hemisphere, there was also significant activation and infiltration of immune cells in the contralateral hemisphere. These results suggested that oHSV infection induced a global inflammatory response in the CNS rather than a localized immune response confined to the tumor. Activation signals such as TNFα are propagated throughout the CNS in response to inflammatory stimuli. Although this study focuses on the antiviral effects of TNFα in response to virus infection many signals such as IL1β, IL6, IFNs, and nitric oxide are released to control oHSV infection (11). These proinflammatory mediators signal in an autocrine and paracrine manner to activate immune cells such as macrophages and enhance their ability to respond to viral infection. oHSV-associated inflammation in the nontumor-bearing hemisphere and surrounding healthy brain parenchyma has not been well studied. These observations may have implications in the treatment of brain tumors to resist caspase-mediated cell death (47). Consistent with microglia and macrophage activation in coculture studies and found both macrophages and microglia reduced virus replication in glioma cells. Together these results confirmed the antiviral capabilities of these cell types in modulating oHSV replication in vivo. These data also support previous studies that identify the antiviral activity of macrophages and microglia against wild-type herpes simplex virus 1 (HSV-1) infections (34, 35).

In this study, we identified TNFα as a major macrophage/microglia–secreted factor that reduces oHSV replication. TNFα is a pleotropic cytokine important for the recruitment and activation of immune cells. Macrophages and microglia are also major producers of TNFα. TNFα is known to limit wild-type HSV replication in the CNS, and it has previously been shown to mediate antiviral effects in studies with wild-type vesicular stomatitis virus, adenovirus-2, encephalomyocarditis virus, HSV-1, HSV-2, respiratory syncytial virus, and influenza through a variety of mechanisms (29, 36–43).

Although TNFα is detrimental to virus replication, TNFα signaling in cancer cells can result in increased tumor cell growth, angiogenesis, invasion, and progression (44–46). Higher levels of the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1 as well as decreased levels of apoptotic proteins such as BAX are commonly observed in recurrent GBM and demonstrate the ability of these tumors to resist caspase-mediated cell death (47). Consistent with
these published studies, we observed that TNFα was not toxic to uninfected GBM cells in vitro. In infected glioma cells, however, we observed that TNFα activated the extrinsic apoptotic pathway resulting in premature cell death, reduced virus replication, and decreased antitumor efficacy. The precise mechanism of how the combination TNFα with oHSV induces apoptosis is unclear. Although HSV-1 has been shown to inhibit apoptosis, previous work has demonstrated the inability of HSV-1 to prevent apoptosis in infected cells exposed to environmental stimuli such as TNFα (48). TNFα ‐ induced cell death was found to be cell ‐ type dependent, and in the case of glioma, this process may depend on the expression of pro‐ and antiapoptotic proteins within the cells.

Oncolytic HSVs expressing TNFα have previously been tested for their ability to enhance oHSV antitumor efficacy (49). In these studies, TNFα ‐ expressing viruses did not enhance antitumor efficacy in an immune ‐ competent lymphoma model compared with a control oHSV that did not express TNFα. In addition, in human squamous carcinoma xenografts, the antitumor efficacy of an oHSV ‐ expressing high levels of TNFα was significantly less than an oHSV ‐ expressing low levels of TNFα. In support of our findings, these results suggest that TNFα elicits strong antiviral responses that may be detrimental to oncolytic HSV therapy.

Although TNFα blockade lead to increased virus propagation, its effect on toxicity in the context of HSV ‐ 1 infections is not clear. Both virus ‐ mediated and immune ‐ mediated mechanisms contribute toward the pathology of HSV ‐ 1 infections. In studies with mice infected with wild ‐ type HSV ‐ 1, TNFα ‐ knock ‐ out mice had higher virus titers and were more susceptible to fatal HSV encephalitis than wild ‐ type mice. These results highlight the protective, antiviral functions of TNFα (29, 36). Although TNFα is important for controlling virus replication, high levels of TNFα have also been shown to induce blood ‐ brain barrier disruption leading to increased inflammation (50). Interestingly, HSV ‐ 1 ‐ infected mice treated with TNFα ‐ blocking antibody showed reduced signs of viral encephalitis and lived longer than those treated with virus alone (51). Thus, a transient blockade of TNFα during virotherapy could increase virus replication and reduce neurotoxicity due to acute inflammation while still allowing for an immune response to eventually clear the infection. Importantly, in our studies, we observed no toxicity associated with TNFα antibody administration in combination with our attenuated, oncolytic virus.

Radio ‐ and chemotherapy also induce the production of cytokines such as TNFα (52–54). Oncolytic virotherapy for GBM is often administered following tumor resection and concurrently with radio ‐ and chemotherapy. As a result, patients may benefit from the transient use of TNFα inhibitors before oHSV administration to enhance oncolytic tumor cell killing and reduce CNS inflammation. The TNFα inhibitors etanercept, adalimumab, certolizumab, and golimumab are currently FDA approved for a variety of diseases and could be readily used in oHSV clinical trials. These inhibitors may be more effective than general immune suppressants, such as high ‐ dose myeloablative CPA, which can have significant toxicities in patients. The combination of oHSV with TNFα inhibitors could enhance virus replication, reduce TNFα ‐ driven tumor proliferation, angiogenesis, and invasion, as well counter the negative effects of chemotherapy/radiotherapy ‐ induced inflammation. This transient inhibition of TNFα could then be removed to allow for the activation of long ‐ term, antitumor immune responses that may be more potent due to increased virus ‐ mediated cell killing and antigen release. In subcutaneous and intracranial tumor studies, we found that the inhibition of TNFα secreted by the tumor microenvironment significantly enhanced virus replication and therapeutic efficacy. In these experiments, we used a TNFα ‐ blocking antibody because the current FDA approved TNFα inhibitors are antibody based. Although the integrity of the blood ‐ brain barrier is disrupted in glioblastoma, the ability of therapeutic antibodies to cross the blood ‐ tumor barrier (BBB) is thought to be limited (55, 56). Although we observed up to 9 ‐ fold increases in viral luciferase expression in subcutaneous tumors, the therapeutic effect in the intracranial tumor studies was more modest. In addition to antibody penetration into the brain tumor microenvironment following oHSV therapy, we hypothesize that the increase in animal survival may have also been through the ability of the antibody to bind TNFα in the serum following oHSV therapy. The future development of specific, soluble TNFα inhibitors that better penetrate the BBB may further increase the antitumor efficacy we observed. These experiments support the future use of TNFα inhibitors in combination with oHSV for GBM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported in part by: NIH grants R01NS064607, R01CA150153, P30NS045758, and P01CA163205 (to B. Kaur) and Pelotonia Fellowships (to A.C. Jaime ‐ Ramirez and S. Dubin).

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Received December 11, 2014; revised February 26, 2015; accepted March 24, 2015; published OnlineFirst March 31, 2015.

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Clinical Cancer Research

The Impact of Macrophage- and Microglia-Secreted TNFα on Oncolytic HSV-1 Therapy in the Glioblastoma Tumor Microenvironment


Clin Cancer Res  Published OnlineFirst March 31, 2015.

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doi:10.1158/1078-0432.CCR-14-3118

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