TGFβ Treatment Enhances Glioblastoma Virotherapy by Inhibiting the Innate Immune Response

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Abstract

Oncolytic viruses, including oncolytic herpes simplex virus (oHSV), have produced provocative therapeutic responses in patients with glioblastoma, the most aggressive brain tumor. Paradoxically, innate immune responses mediated by natural killer (NK) cells and macrophages/microglia appear to limit oHSV efficacy. Therefore, we investigated whether pretreatment with an immunosuppressive cytokine, TGFβ, might reverse these effects and thereby potentiate oHSV efficacy. TGFβ treatment of NK cells rendered them less cytolytic against oHSV-infected glioblastoma cells and stem-like cells in vitro. Furthermore, TGFβ treatment of NK cells, macrophages, or microglia increased viral titers of oHSV in cocultures with glioblastoma cells. In a syngeneic mouse model of glioblastoma, administering TGFβ prior to oHSV injection inhibited intracranial infiltration and activation of NK cells and macrophages. Notably, a single administration of TGFβ prior to oHSV therapy was sufficient to phenocopy NK-cell depletion and suppress tumor growth and prolong survival in both xenograft and syngeneic models of glioblastoma. Collectively, our findings show how administering a single dose of TGFβ prior to oncolytic virus treatment of glioblastoma can transiently inhibit innate immune cells that limit efficacy, thereby improving therapeutic responses and survival outcomes. Cancer Res; 75(24); 1–10. ©2015 AACR.

Introduction

Glioblastoma is the most common and aggressive primary brain tumor in adults (1). The current standard treatment for glioblastoma consists of surgical resection followed by radiotherapy and chemotherapy. However, even with this multipronged approach, the median overall survival of patients with glioblastoma is only 14.6 months due to the highly infiltrative nature of glioblastoma that prevents effective resection (2). Therefore, there is an urgent need to develop novel and effective therapies for this devastating malignancy. Oncolytic viruses are viruses genetically engineered to selectively replicate in tumor cells and trigger tumor cell lysis while sparing normal cells (3). Importantly, this activity is also associated with the enhancement of antitumor immune responses, introducing the potential for extended disease control (4, 5). Oncolytic herpes simplex viruses (oHSV) have been shown to be effective for the treatment of various cancers especially when combined with other reagents, and an oHSV-expressing granulocyte macrophage colony-stimulating factor has demonstrated improvement in durable response rates with a tolerable safety profile in phase III malignant melanoma trials (6). Oncolytic viruses have attracted particular attention as distinctive antiglioblastoma biologic agents, due not only to the relatively restricted localization of glioblastoma in the brain but also to the fact that the surrounding normal cells are postmitotic and thus less susceptible to nonselective viral infection and lysis (7). However, the host innate immune response to oHSV has been shown to impair efficient virus replication and spread within tumor tissues following initial infection, which results in compromised therapeutic efficacy of oHSV against glioblastoma (8, 9). We previously demonstrated that administration of oHSV in the brain induced rapid recruitment and activation of natural killer (NK) cells, which substantially increased viral clearance and limited antitumor efficacy of oHSV in both athymic and immunocompetent mouse models (10). In addition, oHSV-activated NK-cell depletion prolonged overall survival of glioblastoma-bearing mice in a xenograft U87DeltaEGFR (U87dEGFR) model and a syngeneic 4C8 model (10). Suppression of initial innate antiviral defense responses is thus predicted to augment virus replication and tumor lysis, prior to eventual tumor clearance by multiple mechanisms, including later stage host antitumor immune...
responses. Therefore, we hypothesized that temporary or transient inhibition of innate immune responses would enhance the efficacy of oHSV in the treatment of glioblastoma.

The cytokine TGFb1 is secreted by a variety of cells and can exert multiple effects, but in general, it produces cell growth inhibition and apoptosis via transcriptional induction of genes such as the cyclin-dependent kinase inhibitors p15 and p21 (11, 12). Importantly, it plays a critical role in dampening innate immune responses. The immunosuppressive properties of TGFb1 motivated us to explore whether a single pretreatment dose prior to oHSV treatment can temporarily inhibit the anti-oHSV innate immune responses to augment anti-glioblastoma efficacy. In the present study, we found that pretreatment of glioblastoma-bearing mice with a single dose of TGFb1 prior to oHSV administration created a temporary immunosuppressive window that allowed oHSV to replicate and propagate efficiently in the glioblastoma cells, maximizing anti-glioblastoma therapy efficacy and prolonging mouse survival.

Materials and Methods

Cell culture

Vero, GB30/EGFR, and U251 cells were maintained in DMEM supplemented with 10% FBS. Monkey kidney epithelial derived Vero cells were obtained in April 2005 from Dr. E Antonio Chiocca [Ohio State University (OSU), Columbus, OH]. GB30, GB1123, GB84, and GB157 neurospheres, also from Dr. Chiocca (received 2012), were maintained as tumor spheres in neurobasal medium supplemented with 2% B27, human EGF (20 ng/mL), and bFGF (20 ng/mL) in low-attachment cell culture flasks.

Vero cells have not been authenticated since receipt. GB30-EGFR, GB30, and U251 cells were authenticated by the University of Arizona Genetics Core via STR profiling on January 2015. Murine BV2 microglia were maintained in DMEM supplemented with 2% FBS. BV2 cells were received in January 2009 from J. Godbout (OSU). Murine RAW264.7 macrophages, received in June 2010 from Dr. Susheela Tridandapani (OSU), were cultured in RPMI supplemented with 10% FBS. Murine BV2 and RAW264.7 cells have not been authenticated since receipt. Human NK cell line NK-92 was purchased from ATCC and has been authenticated with STR profiling. Human primary NK cells were isolated from peripheral blood leukopacks of healthy donors (American Red Cross, Columbus, OH) as described previously (13). Human NK cells were maintained in RPMI-1640 supplemented with 20% FBS and 150 IU/mL recombinant human (rh) IL2 (Hoffman-La Roche Inc.). All cells were incubated at 37°C in an atmosphere with 5% carbon dioxide and maintained with penicillin (200 U/mL) and streptomycin (100 μg/mL). All cells are routinely monitored for changes in morphology and growth rate. All cells are negative for mycoplasma.

All above antibiotics and cytokines were purchased from Invitrogen. TGFβ1 was purchased from PeproTech. Pan-TGFβ neutralizing antibody 1D11 was purchased from R&D Systems.

Mice

Six- to 8-week-old nude mice, NOD.Cg-Pkdcre39fl/+)Il2rgtm1Wjl/Szl (NSG) mice, and B6D2F1 mice were purchased from Jackson Laboratories. All animal work was approved by The Ohio State University Animal Care and Use Committee and carried out according to an approved protocol. For survival studies, mice were monitored frequently for glioblastoma disease progression and sacrificed when they became moribund with neurologic impairments and obvious weight loss. For flow cytometric analysis of immunoregulation in the setting of oHSV, unless specified, mice were all sacrificed two days after oHSV injection, and tissues were harvested for immune cell isolation. oHSV used in vivo and in vitro is rQNestin34.5, which was previously described (14).

Cytotoxicity assay

A standard 51Cr release assay was performed as described previously (15). Briefly, target cells were labeled with 51Cr and cocultured with the NK-92 cell line or freshly isolated primary NK cells pretreated with or without TGFβ1 at 20 ng/mL for 24 hours at various effector:target (E:T) ratios in the wells of 96-well V-bottom plates at 37°C for 8 hours. Glioblastoma cell lines or patient-derived glioblastoma stem-like cells as target cells were preincubated with or without oHSV for 30 minutes at 37°C (multiplicity of infection, MOI = 3). Supernatants were harvested and transferred into scintillation vials containing a liquid scintillation cocktail (Fisher Scientific), and the release of 51Cr was measured on Beckman Liquid Scintillation Counter LS-6500 (Beckman Coulter). Target cells incubated in complete medium or 1% SDS were used to determine spontaneous or maximal 51Cr release, respectively. Percentage of specific cell lysis was calculated using the standard formula: 100 × (cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release).

Real-time reverse transcription PCR

To detect TNFα, IFNγ, and NOS2 mRNA expression in brain tissues, RNA was extracted from brain samples with the RNeasy Mini Kit (Qiagen) and quantified with NanoDrop (Thermo Fisher). Reverse transcripts were produced using M-MLV reverse transcriptase (Invitrogen), and real-time PCR was conducted with SYBR Green PCR Master Mix (Life Technologies). The PCR primers were described previously (10). PCR reaction parameters were 95°C for 10 minutes, 40 cycles at 95°C for 10 seconds, and 60°C for 60 seconds, followed by 72°C for 10 minutes for final extension.

Coculture virus replication assay

A total of 5 × 105 U251 cells were seeded in wells of 6-well plates with 2% FBS medium and incubated at 37°C overnight. Three milliliter DMEM media supplemented with 0.05% FBS containing oHSV at an MOI of 3 was added to the wells after media aspiration and was incubated at 37°C for 20 minutes. After removal of supernatants and washing with DMEM, the cells (BV2, RAW264.7 or NK-92 cells) pretreated with TGFβ1 or untreated control cells were added (1 × 106 cells per well). After incubation for 12 hours at 37°C, both media and cells were collected for virus titer assays as reported previously (16).

In vivo testing of TGFβ1 effects on oHSV therapy using the orthotopic human GB30 xenograft model and the 4C8 syngeneic model

GB30 human glioma stem–like cells were retrovirally transduced with Pinco-pGL3-luc/GFP virus expressing firefly luciferase (FFL). GFP-positive cells were sorted using a FACSAria II cell sorter (BD Biosciences) and were designated “GB30-FFL” cells. On day 0, 40 nude mice were anesthetized and fixed in a stereotactic apparatus, a burr hole was drilled 2 mm lateral and 1 mm anterior to the bregma to a depth of 3.25 mm, and 5 × 104 GB30-FFL cells were injected under the bregma to a depth of 3.25 mm, and 5 × 104 GB30-FFL cells
in 2 μl Hank's Buffered Salt Solution (HBSS) were implanted. On day 7, the mice were divided into four groups. Mice from the TGFB1 and oHSV combination group were intravenously injected with TGFB1 (1 μg in 200 μl PBS per mouse). Mice from the combination of 1D11 and oHSV group were intravenously injected with 1D11 pan-TGFβ neutralizing antibody (5 μg in 200 μl PBS per mouse), and mice from the oHSV-only group and the HBSS group were intravenously injected with 200 μl PBS. On day 8, all mice from the TGFB1 and oHSV combination, the 1D11 and oHSV combination, and the HBSS-only groups were intratumorally injected with 2 × 10^5 pfu oHSV in 3 μl HBSS, and mice from HBSS group were intratumorally injected with 3 μl HBSS. Mice were monitored daily and euthanized when moribund. Ten days after inoculation of GB30-FFL cells, the mice were intra peritoneally infused with o-luciferin (150 mg/kg body weight; Gold Biotechnology), anesthetized with isoflurane, and imaged using In Vivo Imaging System (IVIS-100, PerkinElmer) and analyzed with live image software (PerkinElmer). For the syngeneic model, 10^5 4C8 murine glioma cells were intracranially injected to each B6D2F1 mouse in the same way as in the GB30-FFL model.

NK-cell depletion in GB30-bearing athymic nude mice and 4C8-bearing B6D2F1 mice

Twenty-five nude mice were intracranially implanted with 5 × 10^6 GB30-FFL cells, as described above, on day 0. On day 8, the mice in the NK-cell depletion groups (asialo + oHSV and asialo + TGFB + oHSV) were intraperitoneally injected with 50 μl anti-asialo GM1 antibody (Wako) combined with 50 μl distilled water. The mice in other groups were intraperitoneally injected with 100 μl distilled water. On day 9, mice in the TGFB + oHSV group and the asialo + TGFB + oHSV group were intravenously injected with TGFB1 (1 μg in 200 μl PBS per mouse). Mice in other groups were intravenously injected with 200 μl PBS. On day 10, mice in the HBSS group were intratumorally injected with 3 μl HBSS, the other mice were intratumorally injected with 2 × 10^5 pfu oHSV in 3 μl HBSS. Mice were monitored daily and euthanized when moribund. Fourteen days after inoculation of GB30-FFL cells, the mice were imaged and analyzed as aforementioned. Survival data were analyzed only after all mice were euthanized. For the syngeneic model, 4C8 murine glioma cells were intracranially injected to each B6D2F1 mouse in the same way as in the GB30-FFL model.

Flow cytometry

Murine mononuclear cells from oHSV-infected brains were isolated as previously described (17). To obtain splenocytes, spleens were collected and homogenized through a 70-mm strainer. Erythrocytes were lysed using RBC lysis buffer (Biolegend). Cells isolated from either brains or spleens were treated with Fc Block antibody (anti CD16/32, BD Biosciences). Cells were stained with mouse-specific immune cell surface markers for 30 minutes at 4°C. The following anti-mouse antibodies were used at a dilution of 1:200: CD3-APC, NK1.1-PE, CD69-FITC, CD27-V450, CD11b-PE, CD45-APC, CD3-PE-Cy7, CD107-APC, CD11b-PerCP-Cy5.5, and IFN-γ-FITC (Biolegend). For CD107a staining, mononuclear cells were cultured in 10% RPMI media with monensin (eBioscience) for 4 hours before cell surface staining. For staining of IFNγ, we treated the cells with Cytofix/Cytoperm (BD) following initial cell surface staining and then performed intracellular staining.

Statistical analysis

Unpaired Student t tests were used to compare two independent groups for continuous endpoints if normally distributed. For non-normally distributed endpoints, such as the in vivo bioluminescence intensity, a Kruskal–Wallis test was used to compare the median of experimental groups. For survival analysis, Kaplan–Meier curves were plotted and compared using a log-rank test. All tests were two-sided. P values were adjusted for multiple comparisons using Bonferroni method. P < 0.05 was considered statistically significant.

Results

TGFB1 inhibits NK-92 and primary NK-cell cytotoxicity against oHSV-infected glioblastoma cells

Our previous study showed that activated NK cells were recruited to oHSV-treated tumors within hours of infection, resulting in substantially compromised oHSV-mediated antitumor efficacy (10). Given that TGFB1 is a well-characterized immunosuppressive factor, we first assessed in vitro whether TGFB1 treatment affects the cytotoxic activity of NK cells against oHSV-infected glioblastoma cells. We preincubated the NK-92 cell line or primary NK cells with or without TGFB1 for 24 hours and infected C57I2-labeled patient-derived glioblastoma stem–like cells or glioblastoma cell lines with or without oHSV for 30 minutes at 37°C. We then combined these cells and assessed target cell (oHSV-infected or uninfected glioblastoma cells) lysis by a standard chromium release assay. TGFB1 treatment significantly impaired the cytotoxic activity of primary NK cells against oHSV-infected or uninfected patient-derived GB30 mesenchymal and GB157 proneural glioblastoma stem-like cells (Fig. 1A), as well as two oHSV-infected or uninfected glioblastoma cell lines, Glis36EGFR and U251 (Fig. 1B). Similarly, after being pretreated with TGFB1, NK-92 cells also showed decreased cytotoxic activity against the oHSV-infected glioblastoma stem–like cells, GB30, GB1123, GB84, and GB157 (Supplementary Fig. S1A) and the oHSV-infected glioblastoma cell lines, Glis36EGFR and U251 (Supplementary Fig. S1B).

TGFβ1 treatment enhances oHSV titers in vitro and in vivo in the presence of glioblastoma cells and innate immune cells

We previously reported that not only NK cells but also macrophages or microglia are recruited to oHSV infection sites to launch an immune response against oHSV and decrease oHSV replication (10, 16, 18). We then tested whether TGFB1 pretreatment could inhibit the antiviral effects mediated by macrophages, microglia, and/or NK cells in vitro and in vivo. The macrophage cell line RAW 264.7 was preincubated with or without TGFB1 for 24 hours and then cocultured with oHSV-infected U251 cells for 12 hours, followed by determination of oHSV titers. In these experiments, the titer of oHSV was significantly increased with TGFB1 pretreatment (Fig. 2A). Similar results were observed using the microglia cell line BV2 (Fig. 2B) or the NK cell line NK-92 (Fig. 2C). We next intracranially implanted U87EGFR cells into nude mice on day 0. On day 7, the mice were treated with TGFB1 or 1D11 (TGFβ neutralizing antibody) by intravenous injection, and on day 8, oHSV was administered intratumorally. On day 10, the mice were sacrificed and oHSV titers in the inoculated hemispheres were measured as described previously (16). Compared with the oHSV-only group, titers in the hemispheres from the combination of TGFB1 and oHSV group increased.
significantly, but titers in the 1D11 plus oHSV combination group decreased (Fig. 2D).

Pretreatment with a single dose of TGFβ1 decreases NK-cell intracranial infiltration and activation in an immunocompetent syngeneic glioblastoma mouse model treated with oHSV

Our previous study showed that NK cells were recruited to the brain and became activated following oHSV therapy (10). We next investigated whether there was any change in NK-cell intracranial infiltration and activation after modulation of TGFβ signaling in a syngeneic glioblastoma model. Here, 4C8 murine glioblastoma cells were implanted into B6D2F1 mice on day 0, followed by an intravenous injection of TGFβ1 or 1D11 on day 7 and an intracranial injection of oHSV or vehicle (HBSS) on day 8 after tumor cell implantation. Mice receiving oHSV showed a significant increase in infiltrating NK cells in the brain relative to mice receiving vehicle (Fig. 3A). However, pretreatment with TGFβ1 resulted in a significant decrease in the total number of intracranially infiltrating NK cells, whereas pretreatment with 1D11 produced a significant increase. We also performed a time course analysis of TGFβ-mediated inhibition of NK-cell infiltration after oHSV injection. The results showed that a single dose of TGFβ led to a significant inhibition of NK-cell intracranial infiltration at days 1 and 2 after oHSV infection. On day 3, TGFβ still produced a moderate, but not statistically significant, inhibitory effect. No inhibition was observed on and after day 5 (Supplementary Fig. S2A). We next characterized the activation status of the intracranially recruited NK cells by evaluating NK-cell activation markers. As expected, oHSV treatment resulted in an increased percentage of CD69-expressing NK cells in inoculated hemispheres significantly following 1D11 pretreatment, whereas TGFβ1 pretreatment dampened CD27 surface expression induced by oHSV treatment (Fig. 3B). The percentage of CD69-expressing NK cells was also significantly decreased with TGFβ1 pretreatment in the setting of oHSV therapy (Fig. 3C). Compared with oHSV treatment alone, expression of CD27 and CD69 appeared to be increased following 1D11 pretreatment, although this increase did not reach statistical significance. IFN-γ-expressing NK cells in inoculated hemispheres significantly decreased after TGFβ1 pretreatment compared with treatment with oHSV alone, and increased significantly following 1D11 pretreatment compared with treatment with oHSV alone (Fig. 3D). At the mRNA level, IFN-γ gene expression in brain tissues was significantly increased with oHSV treatment (Fig. 3E). This increase was effectively blocked by TGFβ1 pretreatment, whereas 1D11 pretreatment enhanced it. We then checked the

**Figure 1.** TGFβ1 pretreatment decreases NK cell cytotoxicity against glioblastoma cell lines and patient-derived glioblastoma stem-like cells. A, cytotoxic activity of primary NK cells or TGFβ1-pretreated primary NK cells against oHSV infected or uninfected patient-derived glioblastoma stem-like cells (GB30 and GB157) was determined using chromium-51 release assay. B, cytotoxic activity of primary NK cells or TGFβ1-pretreated primary NK cells against oHSV infected or uninfected glioblastoma cell lines (Gli36dEGFR and U251) was determined using chromium-51 release assay. Representative data from three independent experiments are shown. *, P < 0.05; **, P < 0.01 (infected glioblastoma cells + NK vs. infected glioblastoma cells + TGFβ-pretreated NK).
status of NK cells in the spleens to determine whether the systemic response paralleled the local brain response. We found that both the percentage of splenic NK cells and expression of the activation/degranulation marker CD107a were decreased after TGFβ1 pretreatment (Supplementary Fig. S3A and Fig. 3F). Percentages of CD27 and CD69 positive NK cells in the spleens also decreased significantly with TGFβ1 pretreatment compared with the oHSV-only group (Supplementary Fig. S3B). Together, these in vivo assays demonstrate that NK-cell numbers and activity are compromised by TGFβ1 treatment prior to oHSV therapy, resulting in attenuated NK cell clearance of oHSV-infected glioblastoma cells.

Macrophages/microglia are suppressed by TGFβ1 treatment in vivo

We then investigated whether TGFβ1 pretreatment affected macrophages/microglia intracranial infiltration in the 4C8 syngeneic glioblastoma mouse model treated with oHSV. Indeed, a significant decrease in the percentage of macrophages/microglia (CD45+CD11b+) was observed in the brain tissues of mice receiving TGFβ1 in conjunction with oncolytic virus therapy (Fig. 4A). Consistent with this result, the mRNA expression levels of two macrophages/microglia activation markers, NOS2 and TNFα (19), were decreased in tumor-bearing hemispheres after TGFβ1 treatment, whereas the opposite effect was observed in mice receiving 1D11 (Fig. 4B and C). A similar decrease in the percentage of macrophages was also detected in spleens from TGFβ1 pretreated mice (Fig. 4D). The time course analysis of TGFβ1-mediated inhibition of macrophages/microglia intracranial infiltration in the presence of oHSV was similar to that of NK cells (Supplementary Fig. S2B).

TGFβ1 pretreatment prior to oHSV administration inhibits glioblastoma tumor growth and prolongs survival of tumor-bearing mice in both xenograft and syngeneic models

To further address the potential application of TGFβ1 treatment in oHSV therapy, we first used the GB30 xenograft glioblastoma model. Patient-derived GB30 stem–like cells expressing firefly luciferase were intracranially implanted into the brains of nude mice. TGFβ1 or 1D11 was administered on
independent experiments, with three mice in each group per experiment. splenic NK cells expressing the degranulation marker, CD107a, following TGF was determined in oHSV inoculated hemisphere in the presence or absence of TGF HBSS without oHSV or 1D11 prior to oHSV, mice that received tumor cell implantation. Compared with control mice receiving day 7, and oHSV or vehicle (HBSS) was injected on day 8 after the tumor cell implantation. Compared with control mice receiving HBSS without oHSV or 1D11 prior to oHSV, mice that received either oHSV alone or TGFβ1 prior to oHSV had significantly reduced tumor growth as determined by bioluminescence imaging (Fig. 5A). This reduction in tumor growth was significantly greater in mice pretreated with TGFβ1 compared with those receiving oHSV alone (Fig. 5B). In agreement with these data, mice treated with TGFβ1 and oHSV survived significantly longer than those treated with oHSV alone (median survival of 26 vs. 19.5 days between the TGFβ1 plus oHSV combination group and the oHSV-only group, P < 0.05; Fig. 5C). In addition, mice receiving oHSV alone survived significantly longer than those pretreated with the TGFβ blocking antibody 1D11 (median survival of 19.5 vs. 16 days between the oHSV-only and the 1D11 + oHSV combination group, P < 0.05; median survival was 16 days for the HBSS group). Results from the 4C8 syngeneic mouse model, shown in Fig. 5D, were consistent with those from the GB30 xenograft model. In the 4C8 model, mice that were pretreated with TGFβ1 before receiving oHSV survived longer than those receiving oHSV alone, whereas survival of mice pretreated with 1D11 was significantly shorter (median survival of 56.5 vs. 42.5 days between the TGFβ1 + oHSV combination group vs. the oHSV-only group, P < 0.05; median survival of 42.5 vs. 25 days between the oHSV-only group and the 1D11 + TGFβ1 combination group, P < 0.05; median survival of HBSS group is 30.5 days).

**TGFβ pretreatment achieved effects similar to NK-cell depletion in oHSV therapy**

To further explore whether the augmented oHSV efficacy resulting from TGFβ1 treatment was associated with dampened...
NK-cell innate immune responses, an asialo-GM1 antibody was injected into athymic mice or B6D2F1 mice to deplete NK cells prior to TGFβ1 treatment, both in our oHSV-treated GB30 luciferase model and our 4C8 model. As shown in Fig. 6A and quantified in Fig. 6B, bioluminescence imaging data indicated that TGFβ1 pretreatment showed effects similar to asialo-mediated NK-cell depletion. Both NK-cell depletion and TGFβ1 treatment resulted in significant improvements in survival in the presence of oHSV therapy. However, there were no significant differences among TGFβ1 treatment, NK-cell depletion, and their combination in either the GB30 xenograft model (Fig. 6C) or the 4C8 syngeneic model (Fig. 6D). Also, virus titers in the hemispheres had no differences among TGFβ1 treatment, NK-cell depletion, and their combination (Supplementary Fig. S4A). Similar data were observed for macrophages/microglia intracranial infiltration (Supplementary Fig. S4B).

**Discussion**

In this study, we determined the effect of TGFβ in innate immune responses to oHSV infection. We also characterized the potential use of TGFβ to inhibit innate immune responses and enhance oHSV therapy for glioblastoma (9, 10, 16). Our in vitro and in vivo studies demonstrate that TGFβ inhibits NK cell and macrophages/microglia intracranial recruitment, activation, and function, thereby permitting enhanced oHSV replication. We demonstrate that the combination of TGFβ with oHSV significantly increases the survival of mice in both syngeneic and xenograft glioblastoma models. The evidence in this study strongly supports that TGFβ regulates innate immune responses in the setting of oHSV therapy and that TGFβ levels can be therapeutically modulated to enhance oHSV efficacy.

Oncolytic virus can selectively infect and reproduce in cancer cells, resulting in cell lysis and virus spread. However, the
recruitment and activation of innate immune cells following oncolytic virus infection are detrimental to antitumor efficacy. NK cells, macrophages, and microglia have all been shown to be deleterious to oHSV replication and spread in tumor cells, as reported by our group and others (10, 16, 20). Using the potently immunosuppressive cytokine TGF-β, here we demonstrate that innate immune responses can be temporarily suppressed to facilitate oHSV replication and spread, thus enhancing antitumor efficacy of the viruses.

Although the host innate immune response can be detrimental to oncolytic virus efficacy, this response also possesses an antitumor capacity that can be harnessed for anti-glioblastoma therapy. NK cells can recognize and eliminate tumor cells while sparing normal ‘self’ cells. Many tumor cells downregulate class I major histocompatibility complex (MHC) molecules and/or upregulate NKRG2D ligands, both of which can promote NK-cell cytotoxicity to these cells (21). Therefore, it is important to balance the anti-virus and antitumor arms of the host innate immune response. There is a consensus that TGF-β acts as an immunosuppressive agent in the case of glioblastoma (22). TGF-β not only downregulates the receptor NKRG2D on NK cells and on CD8+ T cells in patients with glioma but also suppresses expression of the ligands for NKRG2D on glioma cells (23, 24). On the basis of this, we pretreated glioblastoma-bearing mice with a single-dose TGF-β before oHSV administration to create a temporary immunosuppressive window that allowed oHSV to replicate and propagate efficiently in glioblastoma cells and maximize oHSV anti-glioblastoma activity. Our data suggest that TGF-β, used in a proper manner, has the potential to enhance oHSV efficacy against glioblastoma in the clinical setting.

In addition to tumor cells, tumor-associated macrophage/microglia in the glioma microenvironment also secrete high levels of TGF-β (25). Our data indicate that these levels of TGF-β are insufficient to allow effective viral propagation, although they likely have a minor positive effect. We found that the inhibition of basal levels of TGF-β in the tumor microenvironment with a pan-TGF-β neutralizing antibody prior to oHSV injection reduced the efficacy of oHSV therapy. This further supports TGF-β as an important modulator in oHSV efficacy and is consistent with our data showing that transiently increasing TGF-β levels in the tumor microenvironment enhances oHSV-mediated tumor eradication and prolongs survival of glioblastoma-bearing mice.

Innate immunity is the first line of viral defense, and early oHSV expansion is thought to be important for antitumor efficacy. Of note, in our orthotopic glioblastoma model, we observed significant numbers of NK cells and macrophages but few T and B cells in the brain after the intratumoral injection of oHSV (10). These results suggest that early immunosuppression of NK cells and macrophages in particular may improve oHSV therapy. TGF-β has the capacity to inhibit multiple types of innate immune cells, including NK cells, macrophages, and microglia, presenting an ideal cytokine approach to limit innate immune response in the setting of oHSV therapy for glioblastoma. In the GB30 xenograft athymic mouse model, which lacks an adaptive immunity arm, we found that the administration of TGF-β enhanced oHSV therapy to glioblastoma. However, the role of the adaptive immune response in oHSV therapy for glioblastoma cannot be examined in this model. It has been reported that in certain circumstances, an adaptive immune response to oncolytic virus in the treatment of glioblastoma can occur (26). Importantly, in the immunocompetent 4C8 glioblastoma mouse model, we still observed enhanced survival of oHSV and TGF-β-treated mice. These data strengthen our original hypothesis that TGF-β is an important modulator in oHSV therapy.
Further investigation is warranted to explore the clinical application rather than a short-term exposure with exogenous TGFβ as performed here. The tumor-promoting role of TGFβ in glioblastoma is significant when TGFβ is administered alone (27, 28). We did not observe a significant tumor-promoting role (27, 28). We did not observe a significant tumor-promoting role when TGFβ was administered alone (Supplementary Fig. S5). Our speculation for this paradox is that the tumor-promoting role of TGFβ may only be derived from long-term exposure in the tumor microenvironment rather than a short-term exposure with exogenous TGFβ, as performed here.

In conclusion, we demonstrate that TGFβ plays a role in the interactions among tumor cells, oncolytic virus, and host immune responses and that modulation of TGFβ levels can be harnessed to improve the efficacy of oncolytic virus therapy. Further investigation is warranted to explore the clinical application of this novel strategy to improve the efficacy of oncolytic viral therapy for patients with glioblastoma and potentially other types of cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: J. Han, X. Chen, W.H. Meisen, Q.-E. Wang, M.A. Caligiuri, J. Yu

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Han, X. Chen, B. Xu, J. Yu

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