Preferential Replication of Systemically Delivered Oncolytic Vaccinia Virus in Focally Irradiated Glioma Xenografts

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Translational Significance

Glioblastoma remains a therapeutic challenge and with concurrent chemo-radiotherapy the median survival is 12-14 months. Oncolytic viruses are being evaluated for their efficacy in glioblastoma and systemically delivered oncolytic vaccinia viruses have currently entered clinical trials. Since focal radiotherapy is a mainstay of glioblastoma therapy, it is of clinical relevance how systemically administered oncolytic vaccinia virus could be combined with targeted ionizing radiation for therapeutic gain. Focal irradiation of glioma xenografts resulted in preferential replication of systemically delivered vaccinia virus in the irradiated tumor site compared to non-irradiated tumors. These data provide a rationale for future clinical trials combining systemically injected oncolytic virus with radiotherapy. In this therapeutic paradigm, focal radiotherapy can sensitize irradiated tumor sites for viral oncolysis resulting in enhanced tumor control.

Abstract

Purpose: Radiotherapy is part of the standard of care in high grade gliomas but outcomes remain poor. Integrating oncolytic viruses with standard anti-cancer therapies is an area of active investigation. The aim of this study was to determine how tumor targeted ionizing radiation can be combined with systemically delivered oncolytic vaccinia virus.

Experimental Design: U-87 glioma xenografts were grown subcutaneously or orthotopically. Oncolytic vaccinia viruses GLV-1h68 and LIVP 1.1.1 were injected systemically and ionizing radiation was given focally to glioma xenografts. In a bilateral tumor model, glioma xenografts were grown in both flanks, oncolytic vaccinia was injected systemically and radiation was delivered specifically to the right flank tumor, while the left flank tumor was shielded. Viral replication and tumor regression, after systemic injection, was analyzed and compared in irradiated and non-irradiated glioma xenografts.

Results: Systemically administered oncolytic vaccinia virus replicated to higher titers in pre-irradiated U-87 xenografts compared to non-irradiated glioma xenografts. This increased oncolytic viral replication correlated with increased tumor xenograft regression and mouse survival in subcutaneous and orthotopic U-87 glioma models compared to monotherapies. The ability of focal ionizing radiation to mediate selective replication of oncolytic vaccinia was demonstrated in a bilateral glioma model in which systemically administered oncolytic vaccinia replicated preferentially in the irradiated tumor compared to the non-irradiated tumor in the same mouse.

Conclusion: These findings demonstrate a potential clinical role of focal IR in sensitizing irradiated tumor sites for preferential vaccinia virus mediated oncolysis.

Introduction

High grade gliomas remain a therapeutic challenge with a median survival of 12-14 months with conventional therapies of surgical resection followed by chemo-radiotherapy [1]. The resistance of high grade gliomas to standard therapies has resulted in testing
oncolytic viruses to improve tumor control. Oncolytic viruses are replication-competent viruses that selectively infect and destroy tumor cells [2, 3] while sparing surrounding normal tissue. Multiple viruses that selectively replicate in cancer cells and achieve an acceptable safety profile have been identified and initial clinical trials have been completed in high grade gliomas demonstrating the feasibility and safety of oncolytic viral therapy [4-6].

However, a major issue in the development of clinically safe viral oncolytics is the therapeutic tradeoff of efficacy for safety. Preferential tumor replication of oncolytic viruses has primarily been achieved by deletion of viral genes that hampers their replication efficiency [3, 7]. One mechanism to augment the replication of oncolytic viruses is to combine them with standard anti-cancer therapies such as chemotherapy and radiotherapy [7-11]. The cellular response to ionizing radiation (IR) provides a conducive environment for viral replication by upregulation of genes and activation of stress pathways. Since IR is given focally to targeted tumor tissue, studies published to date have primarily evaluated the combination of IR and intra-tumoral oncolytic viral delivery. It was shown that IR can be combined with various intra-tumoral delivered oncolytic viruses to increase viral replication and enhance tumor xenograft regression [12-17]. However, intra-tumoral injections of replication-competent viruses have limited viral distribution within tumor xenografts. To improve viral spread within tumors, oncolytic viruses have been delivered intravascularly [18, 19].

Recently, vaccinia viruses (VACV) have been evaluated for their oncolytic potential [20, 21]. VACV is large double stranded DNA virus in the poxvirus family. Oncolytic VACV has several advantages over other oncolytic viruses. It is unique among DNA viruses in that it replicates exclusively in the cytoplasm to minimize the risk of integrating within the host genome. From a safety issue, VACV has an excellent track record in humans as an agent for immunization against smallpox. VACV also have a large cloning capacity. Oncolytic VACV have demonstrated anti-tumor efficacy in multiple pre-clinical tumor model systems and have entered clinical trials [22-25]. GLV-1h68 was constructed by inserting three expression cassettes into the $F14.5L$, $J2R$ (thymidine kinase), and $A56R$ (hemagglutinin) loci of the viral genome of the LIVP strain of VACV [23]. It has been shown to selectively replicate in cancer cells compared to non-transformed cell lines. In pre-clinical animal tumor models systemically delivered GLV-1h68 replicates in tumors with resultant tumor regression. Intravenous injection of GLONC-1 (clinical grade GLV-1h68) is currently in clinical trials (ClinicalTrials.gov Identifier NCT00794131 and NCT01443260). Here we studied the interaction of systemically delivered oncolytic VACV with focal IR for gliomas. We show that systemically delivered GLV-1h68 in combination with focal IR resulted in improved tumor growth delay and survival. The interaction of IR and oncolytic VACV was not restricted to GLV-1h68, but was also seen with a less attenuated isolate of VACV, LIVP 1.1.1. LIVP 1.1.1 is a plaque purified isolate of the non-attenuated LIVP strain of VACV. Sequencing LIVP 1.1.1 demonstrates it also has a deletion in VACV thymidine kinase gene.

We show for the first time that systemically delivered virus had increased levels of viral replication in focally irradiated tumor xenografts compared to non-irradiated glioma xenografts. In a bilateral animal tumor model, where oncolytic vaccinia virus was delivered systemically to infect both tumors equally and focal IR was delivered to one...
tumor one day prior to virus administration, the irradiated glioma xenograft had higher levels of viral gene expression and viral replication. These data establish a novel use of focal tumor irradiation with systemically delivered oncolytic viruses that takes advantage of the physical delivery properties of radiotherapy. In such a therapeutic paradigm, IR can serve as a means to “radio-paint” tumor areas for targeted enhancement of viral replication and tumor cell kill of systemically administered oncolytic viruses.

Materials and Methods

Cell Culture and VACV Constructs
African green monkey kidney fibroblasts (CV-1) and human U-87 glioma cells were obtained from ATCC. CV-1 and U-87 cells were cultured in DMEM supplemented with 10% FBS. The recombinant, triple mutant VACV GLV-1h68 has been previously described [23]. Briefly, three expression cassettes encoding for Renilla luciferase Aequoria GFP fusion protein, β-galactosidase and β-glucuronidase were inserted into the F14.5L, J2R and A56R loci, respectively, of the viral genome of the LIVP strain. LIVP 1.1.1 is a less virulent wild-type isolate of the LIVP strain (Chen et al., unpublished data).

Viral Proliferation Assay
Standard viral plaque assays were used to quantify viral replication [23]. Infected cells were harvested in triplicate at 24, 48 and 72 hours post infection (hpi). Viral titers were determined in duplicates by plaque assay using serial dilutions on CV-1 monolayers.

Subcutaneous U-87 Glioma Xenografts
Mice were cared for in accordance with approved protocols by the Institutional Animal Care and Use Committee of LAB Research International, Inc. and Explora Biolabs (San Diego Science Center). Five- to six-week old male Hsd:athymic Nude-Foxn1nu (Harlan) were implanted subcutaneously (s.c.) with 5 x 10⁶ U-87 cells (in 100 µl PBS) into the right or bilateral flanks. Treatment started when tumors were 200 – 300 mm³. GLV-1h68 or LIVP 1.1.1 was administered systemically by retro-orbital (r.o.) inoculation of 2 x 10⁶ plaque-forming units (pfu) in 100 µl PBS at day 0. For irradiation, mice were anesthetized by intraperitoneal (i.p) ketamine (3 mg/mouse) and xylazine (0.2 mg/mouse). The body was shielded with lead except for the tumor bearing hind limb, blocking 95% of the given dose as determined by RadCal device. IR was delivered using a RadSource RS 2000 radiator (Rad Source Technologies, Inc.). Tumor growth was measured twice a week and tumor volume was calculated as 0.5 x (length-5) x width x height. Fractional tumor volume (FTV) was defined by tumor volume at each time point divided by initial tumor volume. Bodyweight was measured as net body weight (Bodyweight – Tumor volume/1000) to exclude tumor mass.

Orthotopic U-87 Glioma Xenografts
For the intracranial glioma xenografts athymic nude mice were stereotactically implanted with 1 x 10⁵ U-87 cells. Mice were anesthetized and fixed using a small animal stereotactic frame (David Kopf Instruments). Cells were inoculated into the right frontal lobe. Mice were reversed with Antisedan (Altipamezole) (0.5 mg/kg) and analgesia (Burprenophine) (0.1 mg/kg) was administered. Five days after cell implantation, GLV-
1h68 or LIVP 1.1.1 was delivered systemically by r.o. injection of 2 x 10^6 pfu in 100 µl PBS. In irradiated mice, the irradiated target volume was the entire cranium.

**Expression of Virus Encoded Marker Genes GFP and Luciferase in Tumors**

GLV-1h68 GFP expression within tumors was monitored under UV light using a stereo fluorescence macroimaging system (Lightools Research). GFP expression was scored using a four point system: 0) no GFP signal, 1) one spot, 2) two or three local spots, 3) >3 spots and 4) diffuse signal. For GLV-1h68 luciferase expression, 5 µl coelenterazine (0.5 µg/µl) in 95 µl PBS was given by tail vein injection. Photon emission was recorded for 1 min using the Argus100 Low Light Imaging System (Hamamatsu, Bridgewater, N.J.).

**Tumor Vessel Permeability in Non-irradiated and Irradiated Glioma Xenografts**

Tumor vessel permeability was analyzed using an Evans Blue Dye assay. U-87 glioma xenografts were grown s.c. as described above. Irradiated tumor xenografts received a focal dose of 6 gray (Gy) and 24 h post irradiation mice were injected with 0.1 ml of 1.5% Evans Blue Dye (Sigma) via retro-orbital inoculation. The dye was allowed to circulate for 45 min followed by perfusion with 20 ml PBS. Tumors were harvested and placed in 1 ml of N, N- Dimethylformamide (Sigma) per 0.1 g tissue. Dye extraction was performed at 55 °C for 72 h and absorbance was quantified at 620 nm. Values were normalized to dye extracted from the spleen of the same mouse.

**Vaccinia Viral Titers in Tumor Xenografts and Immune-Related Profiling**

Tumors were excised and placed in two volumes of homogenization buffer (50 mM TrisHCl, 2 mM EDTA) supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics). Tumors were homogenized using a MagNA Lyser (Roche Diagnostics). After three freeze and thaw cycles, the supernatants were collected by centrifugation. Viral titers were measured by standard plaque assay on CV-1 cells. In addition, samples (n=2 per group) were analyzed for immune-related protein antigen profiling by Multi-Analyte Profiles (Rules Based Medicine) using antibody-linked beads. Results were normalized based on total protein concentration and cut off was set at minimum 1.5 fold increase in treated samples compared to control.

**Histology**

Tumors were fixed with 10% neutral buffered formalin over night, dehydrated and then embedded in paraffin. Five µm sections were stained for VACV as described previously [26]. Sections were examined with low magnification (2x) on a Nikon Eclipse 6600 microscope. Images were taken using the Diagnostics Instruments model 24.4 camera and Metamorph v. 7.7 software.

**Statistical Analysis**

Statistical analysis was performed with SPSS, version 11 (SPSS, Inc.). One-way analysis of variance (ANOVA) was used to compare the tumor volumes among different treatment groups at each time point. The differences between the groups were analyzed with Bonferroni tests when the ANOVA showed an overall significance at a time point. To determine significance between only two treatment groups a two-tailed unpaired t-test
was used (Excel 2007 for Windows). Differences in survival were analyzed using Log-rank method.

Results

Combining GLV-1h68 and IR Increases U-87 Glioma Xenograft Growth Delay. We assessed the efficacy of combining GLV-1h68 and IR in a subcutaneous U-87 xenograft model. U-87 glioma xenografts were grown in the right flank of athymic nude mice. GLV-1h68 was delivered systemically on day 0. In irradiated groups, 6 Gy of focal IR was given to the tumor bearing hindlimb. We used a dose of IR that was therapeutically suboptimal and would result in tumor growth delay without tumor regression. IR was delivered either 1 day prior to or 1 day after systemic viral administration to determine if there was an effect on sequencing IR and oncolytic VACV. Tumor volumes were measured biweekly and plotted as mean fractional tumor volume, Figure 1A. The response of individual glioma xenografts in each experimental group is shown in S1. Mice were weighed once a week to monitor general wellbeing, S2A. Untreated control glioma xenografts grew exponentially and all mice were sacrificed by day 23, secondary to tumor burden. Systemic GLV-1h68 injection alone had minimal effect on tumor growth delay and all animals were sacrificed by day 27, secondary to tumor burden. Delivery of a 6 Gy fraction resulted in an initial tumor growth delay, but then irradiated glioma xenografts grew exponentially. Combining 6 Gy with GLV-1h68 resulted in tumor xenograft growth delay compared to either GLV-1h68 or IR alone. Tumors of mice treated with GLV-1h68 and 6 Gy were significantly smaller (p < 0.05) compared to all other treatment groups by day 27. Sequencing of IR, either before or after systemic viral injection, had a similar effect on U-87 xenograft growth delay. To further quantify the effects of GLV-1h68 and IR on glioma growth delay, we calculated the mean time to reach 10 times the initial starting volume, FTV = 10V(0), for each of the groups, Table 1A. Control U-87 xenografts grew to FTV = 10V(0) by 17.8 days. Systemic GLV-1h68 caused minimal tumor growth delay increasing FTV = 10V(0) by 2 days. Focal 6 Gy caused a 10 day increase in FTV = 10V(0) over control tumors. The combination of systemic GLV-1h68 delivery and focal IR, given either 1 day before or after GLV-1h68 injection, resulted in a 22 day increase of FTV = 10V(0).

Combining GLV-1h68 and IR Increases Survival in Mice Implanted Intracranially with U-87 Glioma Xenografts. We then verified the efficacy of combining GLV-1h68 and IR in an intracranial U-87 xenograft model. U-87 glioma cells were implanted intracranially and GLV-1h68 was delivered systemically on day 5. In irradiated groups, two fractions of 6 Gy were given to the entire cranium on days 4 and 6. Mice were followed for survival, Figure 1B, Table 1B. Median survival for control mice was 25 days post implantation. GLV-1h68 did not increase survival. IR increased median survival by 7 days. Mice treated with combination of IR and GLV-1h68 survived significantly longer (p < 0.01) than all other groups, demonstrated by an increase of median survival by 21 days. The effects of combining GLV-1h68 and IR in the intracranial glioma model corroborated our results in the subcutaneous glioma model. To further characterize the interaction of GLV-1h68 and IR, we then utilized the subcutaneous glioma model.
Irradiation Increases In-situ Expression of GLV-1h68 Encoded GFP and Luciferase in Glioma Xenografts. To investigate if focal IR and systemic viral delivery interacted, we monitored in-situ real time GLV-1h68 reporter gene expression within tumor xenografts from groups treated with systemic injection of GLV-1h68 with or without focal tumor irradiation. GLV-1h68 encodes a Renilla luciferase-GFP fusion protein. Viral GFP expression was monitored within the tumor xenografts and scored on a 4 point scale on days 7 and 14 post systemic viral delivery. Tumor xenografts from control mice did not have any detectable GFP expression. At one week post systemic injection of GLV-1h68 alone, U-87 xenografts had focal spots of GFP expression, Figure 2A, S3. In contrast, U-87 xenografts treated with 6 Gy focal to the tumor 1 day prior to systemic GLV-1h68 injection had more diffuse GFP expression within the tumor xenografts, Figure 2A, S4. Interestingly, when 6 Gy of focal IR was given 1 day after systemic GLV-1h68 injection, GFP expression within the tumor was again focal at day 7 similar to non-irradiated GLV-1h68 injected alone group, Figure 2A, S5. The mean GFP expression is shown in Figure 2B. While pre-irradiation initially had more diffuse viral GFP expression, by day 14 viral GFP expression was equivalent if IR was given before or after viral injection. Importantly, irradiated glioma xenografts had higher viral GFP expression than non-irradiated glioma xenografts. To verify higher viral GFP expression in irradiated tumor xenografts, we quantitated viral luciferase activity. Ten days post viral injection, photon counts from GLV-1h68 luciferase activity in 8 non-irradiated and 8 pre-irradiated U-87 glioma xenografts were measured. As with GLV-1h68 GFP expression, IR treated U-87 xenografts had higher luciferase activity than non-irradiated glioma xenografts, Figure 2C. Mean and median photon counts from GLV-1h68 encoded luciferase increased 2.6 fold and 5.2 fold, respectively, in pre-irradiated U-87 xenografts compared to non-irradiated U-87 glioma xenografts (p < 0.05). These data demonstrated IR interacted with oncolytic GLV-1h68 to increase oncolytic VACV gene expression.

Focal IR Does Not Alter Tumor Vessel Permeability of U-87 Glioma Xenografts. Our studies showed an increase in viral marker gene expression in tumors that received a dose of 6 Gy one day prior to GLV-1h68 administration. To determine if the focal dose of 6 Gy increases permeability of the tumor vasculature at the time of systemic virus delivery we performed an Evans Blue Dye assay. One and a half percent Evans Blue Dye in PBS was injected systemically into U-87 tumor bearing mice one day after focal irradiation of tumors, similar to the time of oncolytic VACV injection. Non-irradiated and irradiated tumors along with spleens were analyzed for vessel permeability. We found that there was no significant difference (p = 0.134) in dye extravasation in non-irradiated versus irradiated tumors. The mean normalized Evans Blue extravasation of control and irradiated tumors is shown in S6. These data suggest, that increased viral replication in irradiated tumors is not due to a higher viral dose that reaches the irradiated tumor initially because of increased permeability of vessels.

Combining IR with the Less Attenuated Oncolytic Vaccinia LIVP 1.1.1 Enhances U-87 Xenograft Delay. We next determined if the interaction of IR with oncolytic vaccinia
was limited to attenuated GLV-1h68, or if IR could interact with a less attenuated oncolytic VACV, LIVP 1.1.1. We first compared the replication of GLV-1h68 and LIVP 1.1.1 in U-87 cells grown in culture. U-87 cells were infected at a MOI of 0.01 with either GLV-1h68 or LIVP 1.1.1 and harvested at 24, 48, and 72 hpi. LIVP 1.1.1 replicated to higher titers in U-87 cells compared to GLV-1h68, Figure 3A. U-87 cells infected at an MOI of 0.01 had 65 fold more infectious particles produced by LIVP 1.1.1 compared to GLV-1h68 by 24 hpi. We then assessed the efficacy of LIVP 1.1.1 and IR in U-87 glioma xenografts. LIVP 1.1.1 was injected on day 0 systemically and 6 Gy of IR was delivered one day prior to viral injection. Mean fractional tumoral volumes are shown in Figure 3B. Mice tolerated treatments well as their bodyweights were stable, S2B. Untreated control tumor xenografts grew exponentially and mice had to be sacrificed by day 20, secondary to tumor burden. Ionizing radiation alone given as a 6 Gy fraction resulted in a growth delay of 7 days. LIVP 1.1.1, given alone, resulted in a tumor growth pattern previously described for oncolytic VACV. LIVP 1.1.1 treated glioma growth paralleled untreated control glioma xenografts until day 20, after which the tumor xenografts began to regress. Interestingly, combining LIVP 1.1.1 with IR resulted in a stronger anti-tumor effect as measured by volumetric tumor regression. In the group given 6 Gy, 1 day before LIVP 1.1.1 injection, 5 of 7 tumor xenografts had a FTV ≤ 1 at day 41. In contrast, none of the 7 tumor xenografts treated with LIVP 1.1.1 alone had a FTV ≤ 1. Pre 6 Gy + LIVP 1.1.1 were significantly smaller (p < 0.05) compared to other groups. We then validated our results in a U-87 orthotopic model as above. LIVP 1.1.1 was injected systemically on day 5 and IR delivered in two fractions of 6 Gy, given at day 4 and 6. LIVP 1.1.1 injection increased median survival by 3 days and irradiation alone increased survival by 7 days over control mice. Mice treated with combination showed a 21 day increase over control mice, S7. These results indicate that the interaction of IR with oncolytic VACV is not restricted to the triple deleted GLV-1h68, but is also seen with the less attenuated isolate of LIVP, LIVP 1.1.1.

**Fractionated IR in Combination with Oncolytic Vaccinia Virus Achieves Similar Glioma Xenograft Regression.** Clinical radiotherapy is primarily delivered in a multifractionated scheme for gliomas, and in above intracranial models we tested the efficacy of IR given in two fractions before and after VACV. To be able to fairly compare the different radiation fractionation schedules, we tested similar biologic effective doses (BED) than in the flank glioma model. Assuming an α/β ratio of 10 for tumor cell sensitivity, 6 Gy given as a single fraction is equivalent to 3.5 Gy given in two fractions (total dose of 7 Gy). For the two fraction IR schedule, 3.5 Gy was given one day before and after LIVP 1.1.1 injection. Ionizing radiation alone, given as a single fraction of 6 Gy or two fractions of 3.5 Gy, produced equivalent growth delays of approximately 7 days, which is as expected since both IR schedules have an equivalent BED for tumor cell kill, Figure 3B. Splitting IR in two 3.5 Gy fractions resulted in similar tumor volume regression as seen with 6 Gy and LIVP 1.1.1 and was statistically significant compared to single treatment groups (p < 0.05). With the split fraction regimen, 6 of 7 tumors had a FTV ≤ 1 at day 41. These data indicate IR can be delivered as a large single fraction or as two smaller fractions in combination with LIVP 1.1.1.
IR Increases Oncolytic Vaccinia Viral Replication and Distribution in U-87 Xenografts. We next determined if IR increased the replication and spread of oncolytic VACV in glioma xenografts. Since LIVP 1.1.1 does not encode reporter genes, we checked viral spread within glioma xenografts by IHC for antibody to VACV protein A27L at 7 days post LIVP 1.1.1 injection. Glioma xenografts from control mice showed no staining for VACV protein A27L, Figure 4A. LIVP 1.1.1 injection alone showed focal areas positive for VACV, Figure 4B. Irradiation alone showed no staining for VACV. Irradiation in combination with LIVP 1.1.1 resulted in a much more diffuse staining pattern for VACV protein A27L, Figures 4C, D. The staining pattern for VACV protein was similar when 6 Gy was given before LIVP 1.1.1 or if IR was given as 2 fractions of 3.5 Gy, 1 day before and after LIVP 1.1.1 systemic injection. Since IR doses used were therapeutically suboptimal resulting in tumor xenograft growth delay, no necrosis was evident on H+E staining in irradiated xenografts compared to control xenografts at day 7. We then quantitated the number of infectious LIVP 1.1.1 viral particles in non-irradiated and irradiated glioma xenografts. IR was given as a 6 Gy fraction one day before LIVP 1.1.1 systemic injection. By day 7, glioma xenografts injected with LIVP 1.1.1 had a mean of 1.8 x 10⁷ pfu/gram tumor and irradiated glioma xenografts had a mean of 12 x 10⁷ pfu/gram tumor. Irradiation resulted in a statistically significant 6 fold increase in infectious viral particle production (p = 0.03), Figure 4E.

IR in Combination with Oncolytic Vaccinia Virus Induces a Strong Proinflammatory Tissue Response in U-87 Xenografts. Since oncolytic VACV replication was enhanced with U-87 tumor xenografts, we then determined how the combination of IR and oncolytic VACV influenced the inflammatory cytokine profile within tumors. U-87 glioma xenografts from control mice and mice treated with LIVP 1.1.1, 6 Gy, or the combination of 6 Gy followed by LIVP 1.1.1, were harvested 7 days post infection and analyzed for the expression of murine cytokines and proteins regulating inflammation Table 2. Radiation alone had minimal effects on the expression of murine inflammatory cytokines. As expected, LIVP 1.1.1 replication in the tumor resulted in increase in inflammatory cytokines, in particular RANTES, GM-CSF, IP-10, lymphotactin, and MIP-1 beta. Interestingly, the combination of 6 Gy followed by LIVP 1.1.1 injection resulted in a robust proinflammatory reaction within the tumor. Four cytokines were expressed greater than 10 fold (MCP-1, IL-18, MCP-3, and IP-10). In addition, the majority of the remaining cytokines profiled were higher in the combined treatment group compared to LIVP 1.1.1 or 6 Gy alone. IL-1 beta was interesting in that it was the only cytokine strongly down-regulated by the combination of 6 Gy and LIVP 1.1.1.

In a Bilateral Glioma Tumor Model, Systemically Delivered Oncolytic Vaccinia Preferentially Replicates in Focally Irradiated Glioma Xenografts. To further pursue whether focal IR could serve to target systemically delivered virus to replicate preferentially within irradiated tumors, U-87 xenografts were grown bilaterally in the flanks of athymic nude mice. GLV-1h68 was injected systemically and 6 Gy was given focally to the exposed right flank tumor. The rest of the mouse including the left flank tumor was shielded with lead to block out > 95% of the dose. As a control, to prove IR was focally delivered, glioma xenograft volumes were measured until the tumors were...
harvested at day 9 for histology and viral titers, **Figure 5A, S8.** In the group treated with systemic buffer injection and focal IR to the right flank, the shielded left flank tumor grew exponentially whereas the exposed irradiated right flank tumor showed tumor growth delay, **S8A.** The difference between the shielded left tumor xenograft volumes and exposed right tumor xenograft volumes was statistically significant by day 9 (p < 0.05). In the group treated with systemic GLV-1h68 alone, both the right and left tumors grew similarly and tumor volumes were not different, **S8B.** In the group treated with systemic GLV-1h68 and focal right flank irradiation, the exposed right flank tumors were significantly smaller than the unblocked left flank tumors (p < 0.05), **Figure 5A.**

In the mice with bilateral flank glioma xenografts, we determined how well systemically delivered GLV-1h68 replicated in the shielded left flank tumor compared to the right flank tumor exposed to 6 Gy. Of the six mice treated with systemic GLV-1h68 and focal IR to the right flank, all six mice had higher GFP expression in the irradiated exposed right flank tumor compared to the shielded left flank tumor. A representative mouse is shown, **Figure 5B.** GLV-1h68 GFP expression was higher in the irradiated right flank tumor (upper flank) compared to the shielded left flank tumor. GLV-1h68 luciferase expression was 2 fold higher in the irradiated flank tumor compared to the contralateral shielded flank tumor. Finally, bilateral xenografts were harvested on day 9 and tumor sections were stained for VACV protein. Similar to viral GFP and luciferase expression, IHC staining revealed a greater distribution of VACV protein in the irradiated right flank tumor compared to the shielded left flank tumor, **Figure 5C.**

**Discussion**

In this series of studies, we have shown how focal IR can be incorporated with systemically delivered oncolytic VACV to increase viral replication and enhance tumor xenograft regression. Focal IR increased replication of systemically injected oncolytic VACV GLV-1h68 and LIVP 1.1.1 in the targeted irradiated tumors compared to non-irradiated glioma xenografts. More importantly, we showed for the first time that focal IR can result in preferential replication of systemically delivered oncolytic viruses in a pre irradiated tumor target compared to non-irradiated tumors using a bilateral murine tumor model system. Interestingly, there was qualitatively a more diffuse GLV-1h68 GFP signal and quantitatively increased GLV-1h68 luciferase activity in the pre irradiated right flank glioma xenografts compared to the shielded left flank U-87 xenografts. To our knowledge, this is the first demonstration that focal IR resulted in preferential oncolytic viral replication in an irradiated tumor xenograft compared to non-irradiated tumor xenograft in the same animal. In this treatment paradigm, systemically delivered oncolytic viruses are not sensitizers for radiotherapy as is the case with systemically delivered chemotherapy. Instead, IR functions as an oncolytic viral sensitizer to promote viral replication within targeted irradiated tumors.

The safety concerns in the use of oncolytic viruses have prompted the generation of oncolytic viruses genetically engineered with multiple mutations [3, 7]. We and others have shown that increased viral attenuation for safety comes at the cost of anti-tumor efficacy. Our data reported here, with LIVP 1.1.1 and GLV-1h68, are further proof of this concept. The less attenuated LIVP 1.1.1 replicated more efficiently in glioma cells
and translated in a more profound U-87 xenograft delay compared to GLV-1h68. One approach to improve the therapeutic efficacy of attenuated oncolytic viruses is to create a more favorable environment for viral replication. Immunomodulation with cyclophosphamide or rapamycin has previously been shown to enhance the efficacy of systemically delivered oncolytic VACV in experimental glioma models [27]. Our results demonstrated that the combination of IR and LIVP 1.1.1 resulted in a robust proinflammatory response. In particular, MCP-1, MCP-3, IL-18, and IP-10 were found within higher levels in the tumor than seen with IR or LIVP 1.1.1 alone. In a pancreatic tumor model, these four cytokines were also observed to be upregulated by GLV-1h68 infection at day 21 and 42 post infection [28]. Immunohistochemistry staining of infected tumors in previous studies demonstrated an intense peri- and intra-tumoral infiltration of mononuclear cells, indicating the activation of innate immune mechanisms [23]. We proposed that tumor regression induced by GLV-1h68 was at least partially mediated through activation of innate immune mechanisms. Our results here show the combination of IR to LIVP 1.1.1 resulted in high expression of these cytokines by as early as 7 days post infection. These results suggest the combination of IR and oncolytic VACV can activate a proinflammatory tumor response. Further studies are currently underway to determine if cytokine expression mediates an inflammatory tumor response or is secondary to enhanced oncolytic VACV replication mediated by IR.

Data presented here indicate that IR has an ability to enhance the replication of oncolytic VACV that is not restricted to a specific viral mutant. Both GLV-1h68 and wild type LIVP 1.1.1 replicated to higher titers in irradiated U-87 xenografts. One explanation for how focal tumor IR can enhance systemically delivered VACV replication in tumor xenografts is that IR increases the vascular permeability in tumors allowing increased extravasation of oncolytic VACV into irradiated tumor xenografts. However, in our experimental tumor model system, we were not able to detect any significant difference in dye extravasation 24 hrs following IR. Thus, focal IR does not appear to result in enhanced oncolytic VACV as a result of IR altering the tumor vasculature. The ability of IR to enhance oncolytic VACV replication also appeared to be specific to tumor cells and not normal tissue. Following focal irradiation of tumor xenografts and LIVP 1.1.1 delivered systemically, there was no increase in viral particles recovered from non-irradiated normal tissue (spleen, liver, lungs and brain) (data not shown). Moreover, when normal tissue was focally irradiated in non tumor bearing mice and GLV-1h68 systemically injected, the irradiated normal tissue had no viral particles detected by viral titration (data not shown). This suggests that IR enhanced VACV replication maintains the tumor selectivity of oncolytic VACV.

To characterize the interaction of oncolytic VACV with IR, we investigated how IR delivery sequence and IR dose influenced oncolytic VACV replication. To determine if a temporal relationship existed with IR and oncolytic VACV, a single 6 Gy fraction of IR was delivered either 1 day before or after systemic viral injection. Both temporal sequences of oncolytic virus and IR enhanced viral replication and tumor xenograft regression. While IR delivered 1 day before GLV-1h68 infection had an earlier peak (day 7) of VACV GFP expression within tumors, by day 14 post infection VACV GFP tumor expression was similar if IR was given 1 day prior to or after GLV-1h68 infection. One explanation for these results is that IR delivered 1 day after VACV replication may
prime cells for infection by progeny virus released by the 1st wave of tumor infecting VACV.

Clinical radiotherapy is delivered in a fractionated scheme for gliomas. When the biological effective dose of IR was held constant, two fractions of 3.5 Gy produced a similar glioma xenograft growth delay as a single 6 Gy fraction alone. When both fraction schemes were combined with LIVP 1.1.1, they both resulted in increased glioma xenografts regression and more diffuse LIVP 1.1.1 spread in U-87 xenografts. Thus, it appears that oncolytic vaccinia virus could be incorporated into either larger hypofraction or more conventionally fractionated IR. The choice of radiotherapy fractionation scheme would be dictated by clinical relevance and surrounding normal tissue constraints.

Here we have demonstrated a rationale and utility of combining focal IR with systemic oncolytic virus administration. Currently, IR is routinely combined with systemically delivered cytotoxic chemotherapies such as cisplatin and temozolomide or targeted agents such as erlotinib and cetuximab. Here we showed that IR can act as a sensitizer for tumor oncolysis by VACV. Our preclinical results have implications in how focal radiotherapy can be combined with systemic oncolytic viral administration for locally advanced tumors. Focal IR to tumors can provide a spatial target for systemically delivered oncolytic virus. Such “radio-painting” of tumors may result in preferential and enhanced oncolysis within the irradiated tumor target while sparing surrounding normal tissue. Furthermore, clinically this may lead to reduction of both viral and radiation dose resulting in safe and efficient tumor regression.

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References


Figure Legends:

Figure 1: Inhibition of Glioma Xenograft Growth by Combination of GLV-1h68 and IR.

A) S.C. U-87 xenografts were grown in flanks of athymic nude mice. Mice were treated with systemic GLV-1h68 and focal 6 Gy IR either one day before or after virus injection. Glioma xenografts were measured twice a week and plotted as mean fractional tumor volume. B) Kaplan Meier survival curves of IC U-87 glioma xenografts. U-87 glioma cells were orthotopically implanted into the brains of nude mice. GLV-1h68 was injected systemically and IR was given in two 6 Gy fractions one day before and after GLV-1h68 injection.
Figure 2: Expression of Viral Encoded *Renilla* Luciferase-GFP Fusion Protein in U-87 Glioma Xenografts Treated with GLV-1h68 and IR. U-87 glioma xenografts were injected systemically with GLV-1h68. IR was given as single fraction of 6 Gy one day before or after viral injection. A) Bright field and fluorescence image of representative mice 7 days post GLV-1h68 injection. B) Tumoral GFP expression was scored on a 4 point system on days 7 and 14 post systemic viral injection. C) Tumoral GLV-1h68 luciferase expression 10 dpi.

Figure 3: LIVP 1.1.1 Replicates More Efficiently in Glioma Cells and Increases Tumor Growth Delay with IR. A) Increased replication of LIVP 1.1.1 versus GLV-1h68 in U-87 glioma cells. U-87 glioma cells were infected with either GLV-1h68 or LIVP 1.1.1 and harvested 24, 48, and 72 hpi. Viral titers were determined on CV-1 monolayer and normalized to the input virus. B) U-87 glioma xenograft growth delay with LIVP 1.1.1 and IR. U-87 glioma xenografts were grown in nude mice and systemically injected with LIVP 1.1.1 on day 0. IR was given as a single 6 Gy fraction one day before LIVP 1.1.1, or in two 3.5 Gy fractions one day before and one day after LIVP 1.1.1 injection. Glioma xenografts were measured twice a week and normalized to the volume at initiation of treatment, FTV.

Figure 4: Combination of LIVP 1.1.1 with IR Results in Increase of VACV in Tumors. A-D) Distribution of LIVP 1.1.1 in non-irradiated and irradiated U-87 glioma xenografts. IHC to VACV in U-87 glioma xenografts harvested 7 days post LIVP 1.1.1 systemic injection. All pictures were taken at 2x magnification. A) Control U-87 xenografts from non-infected mice. B) LIVP 1.1.1 treatment alone. C) 6 Gy one day before LIVP 1.1.1 injection. D) 3.5 Gy one day before and one day after LIVP 1.1.1 injection. E) Infectious LIVP 1.1.1 particles within non-irradiated and irradiated U-87 glioma xenografts. IR was given as a single 6 Gy fraction one day before systemic LIVP 1.1.1 injection. Glioma xenografts were harvested 7 days post LIVP 1.1.1 injection and infectious viral particles were quantitated on CV-1 monolayer.

Figure 5: Preferential Replication of Systemically Delivered GLV-1h68 within the Focally Irradiated Right Tumor as Opposed to the Non-Irradiated Left Tumor in a Bilateral Flank Tumor Model. U-87 glioma xenografts were grown in the bilateral flanks of mice and GLV-1h68 was injected systemically. IR was given focally as single 6 Gy fraction one day before viral injection to the right flank tumor. The remainder of the mouse and the left flank tumor were shielded with lead. A) Serial measurements of fractional tumor volumes of the left and right flank glioma xenografts. B) GLV-1h68 reporter gene expression in a representative mouse with bilateral glioma xenografts. 6 Gy was given focally to the right flank tumor (upper tumor) with the left flank (lower tumor) shielded. Upper left panel: bright field, Upper right panel: GFP expression, Lower left panel: Luciferase activity (photons/minute), Lower right panel: Overlay of luciferase activity photon count with mouse contour. C) IHC to VACV 9 days after GLV-1h68 systemic injection in shielded left flank tumor (left panel) and irradiated right tumor (right panel). Pictures were taken at 2x magnification.
Table 1. Growth Inhibition of Subcutaneously (A) or Intracranially (B) Implanted U-87 Glioma Xenografts

A) Time to Reach 10 Times the Starting Tumor Volume and Growth Delay to FTV = 10 by GLV-1h68, IR, or the Combination.

<table>
<thead>
<tr>
<th></th>
<th>Time to Reach FTV = 10 (Days)</th>
<th>Tumor Growth Delay over Control Tumors (Days)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>17.8</td>
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</tr>
<tr>
<td>GLV-1h68</td>
<td>19.7</td>
<td>1.9</td>
</tr>
<tr>
<td>6 Gy</td>
<td>28.4</td>
<td>10.6</td>
</tr>
<tr>
<td>Pre 6 Gy + GLV-1h68</td>
<td>39.2</td>
<td>21.4</td>
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<tr>
<td>GLV-1h68 + Post 6 Gy</td>
<td>41</td>
<td>23.2</td>
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B) Median Survival for all Groups and Survival Increase over Control Mice.

<table>
<thead>
<tr>
<th></th>
<th>Median Survival (Days Post Implantation)</th>
<th>Survival Increase over Control Mice (Days)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>GLV-1h68</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>2 x 6 Gy</td>
<td>32</td>
<td>7</td>
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<tr>
<td>2 x 6 Gy + GLV-1h68</td>
<td>46</td>
<td>21</td>
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## Table 2. Mouse Immune-Related Protein Antigen Profiling of S.C. U-87 Xenografts

### A) Protein Expression Upregulated

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<thead>
<tr>
<th>Antigen Name</th>
<th>LIVP 111 / Control Ratio</th>
<th>6 Gy / Control Ratio</th>
<th>LIVP 111 + 6 Gy / Control Ratio</th>
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<td>Interferon gamma Induced Protein 10 (IP-10)</td>
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<tr>
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<td>Stem Cell Factor (SCF)</td>
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<td>Endothelin-1 (ET-1)</td>
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<td>Interferon gamma (IFN-gamma)</td>
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<td>Leukemia Inhibitory Factor (LIF)</td>
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<tr>
<td>Growth-Regulated Alpha Protein (KC/GRO)</td>
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<td>Oncostatin-M (OSM)</td>
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<td>1.0</td>
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<td>1.6</td>
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</table>

### B) Protein Expression Downregulated

<table>
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<th>Antigen Name</th>
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<th>6 Gy / Control Ratio</th>
<th>LIVP 111 + 6 Gy / Control Ratio</th>
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<tbody>
<tr>
<td>Interleukin-1 beta (IL-1 beta)</td>
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<td>Tissue Factor (TF)</td>
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### Fold Upregulation and Fold Downregulation

- Fold Upregulation: >1, 2-5, 5-10
- Fold Downregulation: >1, 2-5, 5-10
Clinical Cancer Research

Preferential Replication of Systemically Delivered Oncolytic Vaccinia Virus in Focally Irradiated Glioma Xenografts


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