Intravenous delivery of oncolytic reovirus to brain tumor patients immunologically primes for subsequent checkpoint blockade

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Immune checkpoint inhibitors, including those targeting programmed cell death protein 1 (PD-1), are reshaping cancer therapeutic strategies. Evidence suggests, however, that tumor response and patient survival are determined by tumor programmed death ligand 1 (PD-L1) expression. We hypothesized that preconditioning of the tumor immune microenvironment using targeted, virus-mediated interferon (IFN) stimulation would up-regulate tumor PD-L1 protein expression and increase cytotoxic T cell infiltration, improving the efficacy of subsequent checkpoint blockade. Oncolytic viruses (OVs) represent a promising form of cancer immunotherapy. For brain tumors, almost all studies to date have used direct intralesional injection of OV, because of the largely untested belief that intravenous administration will not deliver virus to this site. We show, in a window-of-opportunity clinical study, that intravenous infusion of oncolytic human Orthoreovirus (referred to herein as reovirus) leads to infiltration of tumor cells subsequently resected as part of standard clinical care, both in high-grade glioma and in brain metastases, and increases cytotoxic T cell tumor infiltration relative to patients not treated with virus. We further show that reovirus up-regulates IFN-regulated gene expression, as well as the PD-1/PD-L1 axis in tumors, via an IFN-mediated mechanism. Finally, we show that addition of PD-1 blockade to reovirus enhances systemic therapy in a preclinical glioma model. These results support the development of combined systemic immunovirotherapy strategies for the treatment of both primary and secondary tumors in the brain.

INTRODUCTION
Therapies targeting T cell inhibitory checkpoint signaling pathways, including programmed cell death protein 1 (PD-1) monoclonal antibodies, have produced unprecedented results in recent years in solid malignancies (1–4). Unfortunately, only a minority of patients benefit, with mounting evidence that tumor response and patient survival are associated with tumor programmed death ligand 1 (PD-L1) expression (5) and preexisting tumor-infiltrating cytotoxic T cells (CD8+) (6). Oncolytic virus (OV) immunotherapy uses wild-type or genetically modified viruses selectively to kill tumor cells and promote tumor-directed innate and adaptive immune responses (7, 8). The first OV to receive U.S. Food and Drug Administration approval was talimogene laherparepvec (T-VEC), after a phase 3 trial demonstrating superior outcomes in patients with advanced melanoma treated with intralesional T-VEC compared to subcutaneous granulocyte-macrophage colony-stimulating factor (GM-CSF) (9). Major challenges remain, including the optimization of combination therapies and routes of virus delivery. In particular, the combination of OV with immune checkpoint blockade deserves attention, because a number of OVs stimulate the secretion of interferons (IFNs) (10, 11), intermediary cytokines in PD-1/PD-L1 expression. Furthermore, OV delivery to tumors can enhance T cell infiltration (11), hence priming the tumor immune microenvironment for immune-mediated therapy when combined with PD-1/PD-L1 axis blockade.

For patients with brain tumors, concerns that the blood-brain barrier (BBB) may inhibit OV delivery have, thus far, limited studies using intravenous administration, notwithstanding the infiltrative and/or multifocal nature of such tumors. A number of OVs, including herpes simplex virus (HSV)–1716 (12–14), HSV-G207 (15), adenovirus-dl1520 (ONYX-015)(16), and reovirus (17, 18), have been trialed in glioma patients by surgical intratumoral or intracavity injection. These techniques require careful patient selection and technically challenging neurosurgery, limiting repeat administration. Yet, the need for effective therapies in this group of patients cannot be overemphasized; median survival for grade IV gliomas [glioblastoma multiforme (GBM)] after tumor-directed surgery and chemoradiotherapy is 14.6 months (19), and those with a single brain metastasis and controlled extracranial
disease survive only 9 to 10 months, despite optimal treatment (20). The clinical trial described herein tested whether intravenous reovirus could infect recurrent high-grade gliomas (HGGs) and metastatic brain tumors in patients and examined the ensuing immunological sequelae, with particular focus upon the tumor microenvironment.

RESULTS

Intravenous-injected reovirus accesses brain tumors in mice

Preclinical experiments confirmed that intravenous reovirus selectively accesses intracranially implanted malignant melanoma in immunocompetent mice, albeit to varying degrees (fig. S1). Reovirus σ3 capsid protein and reovirus RNA were strongly detected after intravenous infusion in mice 1 and 2, suggesting viral genome replication and translation, but were only detectable in extremely low amounts in mouse 3. Lower magnification pictures revealed high reovirus protein expression clustered in small areas of the tumor, with lower expression in a larger number of tumor cells (fig. S1, middle row). Reovirus was not detected in normal peritumor murine brain tissue or phosphate-buffered saline (PBS) control.

Intravenous reovirus associates with multiple peripheral white blood cell subsets in patients

On the basis of the murine experiment results, we recruited nine patients to a phase 1b window of opportunity trial (table S1), where each patient was treated with a single, 1-hour intravenous infusion of 1 × 10^10 TCID_{50} (50% tissue culture infectious dose) reovirus ahead of planned surgical resection of his or her brain tumor. Treatment was well tolerated in all cases, and surgery was undertaken 3 to 17 days after reovirus infusion. The most commonly observed adverse events were lymphopenia (grades 1 to 2 in all nine patients, grades 3 to 4 in six patients) and flu-like symptoms. Median overall survival from the day of reovirus infusion to death was 469 days (range, 118 to 1079 days), which is consistent with the expected survival for this group of patients that have variable cancer diagnoses.

Extending upon findings from our previous study (21), where we demonstrated intravenous reovirus carriage and protection from neutralizing antibody by peripheral blood mononuclear cells (PBMCs), granulocytes, and platelets, we examined white blood cell (WBC) subsets taken mid-reovirus infusion for reovirus RNA by reverse transcription polymerase chain reaction (RT-PCR) (fig. S2A). In addition to granulocytes, we confirmed the association of reovirus RNA with CD14^+ [monocytes, which are pivotal for reovirus cell carriage in mice (22)], CD19^+ (B cells), and CD56^+ [natural killer (NK)/NK T cells] fractions, but viral RNA could not be detected on CD3^+ (T cells) in this subset of trial patients, for whom samples were available. Time course analysis of IFN-α concentrations in patient sera taken before and after reovirus revealed significantly increased IFN-α (P = 0.0153) 2 days after infusion, in comparison to baseline (fig. S2B). This indicates reovirus engagement of pattern recognition receptors, potentially during carriage by peripheral WBCs, resulting in systemic IFN release. Plasma concentrations of other inflammatory cytokines were also increased 2 days after reovirus infusion, relative to preinfusion concentrations (table S2).

Reovirus is detected in resected brain tumors from trial patients

Examination of resected brain tumors by immunohistochemistry (IHC) revealed the presence of reovirus σ3 capsid protein in low amounts in six of nine tumors (Fig. 1A, top, and table S3) and nine of nine tumors by immunogold transmission electron microscopy (TEM) (Fig. 1B). Resected brain tumor specimens from patients outside the trial served as controls. Secondary antibody–only controls for background immunogold staining in trial patient tumors are shown in fig. S3. The vast majority of reovirus protein was localized to tumor cells, with only 0 to 6% localizing to endothelial cells (table S3 and fig. S4). Examination of the specimens by in situ hybridization (ISH) revealed eight of nine tumors to be positive for reovirus RNA (Fig. 1A, bottom), with reovirus RNA being detected in a higher percentage of cells than reovirus σ3 protein in all cases (table S3), consistent with the findings in mice (fig. S1). In comparison, control brain tumors showed no reovirus RNA staining (Fig. 1A, bottom). The presence of reovirus RNA in tumors was further examined by quantitative RT-PCR (qRT-PCR) amplification of the S4 genome segment (encoding σ3), confirming four of the seven available tumor samples to be positive (Fig. 1C). Despite some variation in detection limits for different techniques, together, these data convincingly support delivery of systemically administered reovirus to patient brain tumors.

The distribution of reovirus RNA and protein within tumors was further examined using immunofluorescence (IF) (Fig. 1D for trial tumors and fig. S5A for control). Reovirus RNA was detected in a large proportion of cells, whereas reovirus protein and protein–RNA colocalization were only detected in discrete areas of tumor, suggesting that reovirus protein translation and/or productive infection occurred only in small areas of tumor, at least by the snapshot time point of surgical resection.

The presence of reovirus RNA and protein in tumors correlates with Ki67

The overall proportion of reovirus σ3 protein- and RNA-positive cells within individual tumors varied widely between the nine trial patients (table S3). Because actively dividing cells preferentially support reovirus replication in comparison to quiescent cells (23, 24), we analyzed resected trial patient and control tumors for expression of the proliferation marker Ki67 relative to reovirus protein/RNA (Fig. 2, A and B, and table S4). The amounts of both reovirus σ3 protein and RNA correlated with tumors containing a high proportion of Ki67-positive cells (P = 0.014 for σ3 protein and P = 0.016 for reovirus RNA). However, IF analysis of tumors revealed little coexpression of reovirus RNA and Ki67 (Fig. 2C for trial tumors and fig. S5B for control), potentially because Ki67 staining is restricted to particular phases of the cell cycle (25). Further, IF examination of tumors confirmed the presence of low amounts of reovirus σ3 protein, as was detected by IHC and TEM, and showed reovirus protein to frequently colocalize with tubulin, a key component of reovirus replication factories (Fig. 2D for trial tumors and fig. S5C for control) (26). Together, these results indicate that tumors with a higher proliferation index are more susceptible to reovirus infection but that reovirus protein translation and/or productive infection overall occur at relatively low rates only. In keeping with these observations and in contrast to our previous trial in resected colorectal liver metastases (21), replication-competent reovirus could not be retrieved from any of the nine trial tumors. Analysis of the number of days between reovirus administration and surgery revealed no significant change in reovirus RNA and protein over time from reovirus infusion (fig. S6).

Reovirus treatment increases tumor leukocyte infiltration

We used RNA sequencing (RNA-seq) to compare expression of coding and noncoding transcripts in whole tumor RNA from three GBM
trial patient samples (cases 1, 6, and 7) to that of three control GBM tumors. Given that sample numbers were small, criteria for statistically significant differential gene expression between treatment and control tumors were stringently set as described in Materials and Methods (q < 0.1). Of the 2366 sequenced transcripts, 102 genes were differentially expressed between reovirus-treated and untreated GBM.
Two of these transcripts were CCL3 (mean control group expression = 4.2, mean treatment group expression = 34.3, \(q = 0.0188\)) and CCL4 (mean control group expression = 2.5, mean treatment group expression = 19.2, \(q = 0.0188\)), which both function to recruit CD8+ T cells and other leukocytes to sites of immunization (27). CCL4 protein and a number of other chemokines were higher in trial patient plasma 2 days after reovirus infusion, relative to preinfusion concentrations (table S2). Furthermore, peripheral blood assessment of CD4+ and CD8+ T cell populations revealed increased cell surface expression of intercellular adhesion molecule (ICAM) 2 days after intravenous reovirus infusion, in comparison to baseline expression (Fig. 3A). ICAM expression is up-regulated by inflammatory cytokines, enhancing leukocyte interaction with vascular endothelial cells to enable migration to sites of inflammation (28). In keeping with these observations, IHC analysis of trial patient and control tumors revealed CD3+ T cells in and around blood vessel walls, in virus-treated but not untreated controls, consistent with reovirus-induced chemotaxis of T cells into infected brain tumors (Fig. 3B). Further IHC assessment for tumor-infiltrating cytotoxic T cells (CD8+), which are critical for PD-1/PD-L1-directed immunotherapy (29), revealed their presence in eight of the nine trial patient tumors, four of which showed more staining (2+ or 3+), in comparison to the control cases, where CD8+ T cell

Fig. 2. Correlation of reovirus RNA/protein with proliferating tumor cells. (A) Trial patient IHC tumor sections stained for Ki67 (brown) with indicated percentages of cells positive for Ki67 and reovirus o3 protein (from table S3), showing examples of tumors with high reovirus o3 staining (top) and no reovirus o3 protein staining (bottom). Scale bars, 40 \(\mu\)m. GBM, glioblastoma multiforme. (B) Scatter plot and line of best fit, correlating the percentages of tumor cells positive by IHC for reovirus RNA or o3 protein and for Ki67. (C) Representative tumor sections derived from trial patient 9 (high Ki67; top), trial patient 1 (intermediate Ki67; middle), and trial patient 4 (low Ki67; bottom), showing IF staining for reovirus RNA (blue), Ki67 (red), or their coexpression (yellow, arrows). Scale bars, 40 \(\mu\)m. (D) Representative trial patient tumor IF staining for tubulin (fluorescent red), reovirus o3 protein (fluorescent green), and their coexpression (yellow). Nuclear counterstaining is blue. Scale bars, 40 \(\mu\)m (top and bottom) and 80 \(\mu\)m (middle).
infiltration was detected only in three of the six tumors, all in low amounts (Fig. 3C and table S4).

We also examined tumors for the presence of CD68+ microglia/infiltrating macrophages and found these to be present in higher numbers in tumors from reovirus-treated patients in comparison to controls (fig. S7 and table S4). Very few tumor-infiltrating CD56+ NK cells and CD19+ B cells were found in any tumor. The daily dose of dexamethasone taken by patients within and outside the trial did not appear to correlate with tumor immune cell infiltration in the examined surgical specimens (table S4).

**Genes associated with programmed cell death are more highly expressed in GBM tumors from reovirus-treated patients than in matched controls**

Functional analysis of the differentially expressed genes found by RNA-seq indicated significant enrichment in members of several biological processes, including those governing programmed cell death ($P = 0.0003$), regulation of viral transcription ($P = 0.0000502$), and cytokine activity ($P = 0.0129$) (table S6). Consistent with these RNA expression data and preclinical models (30), IHC analysis of trial HGG samples revealed a higher proportion of tumor cells to be positive for cleaved caspase 3, albeit in a small number of patients, than in controls, suggesting the specific induction of apoptosis within tumors after intravenous reovirus infusion (Fig. 4A and table S4). A similar pattern was observed for the three trial brain metastases in comparison to controls (fig. S8A and table S4).

**PD-L1 expression is higher in tumors resected from reovirus-treated patients than in controls**

We next sought to determine whether reovirus treatment results in the up-regulation of IFN-regulated genes (IRGs). Of the 23,366 genes expressed in our samples, 5031 are IRGs (31), in contrast to 48 of the 102 genes that were differentially expressed between trial and control samples ($\chi^2$ test, $P = 1.28 \times 10^{-8}$).

IFN transcripts and PD-1 messenger RNA (mRNA) were not detected in our analysis, perhaps due to a transient rise and fall in expression before the time point of surgical resection. The expression of PD-L1 was, on average, about twice as high in reovirus-treated patient GBM samples than in controls, but the difference was not statistically significant (1.310 in trial samples versus 0.668 in controls). However, protein analysis by IHC revealed consistent PD-L1 expression in trial HGGs but not in controls (Fig. 4B and table S4), and a similar pattern was observed for brain metastases (fig. S8B and table S4). Of the two melanoma metastases in the trial, case 9 (which stained most intensely for Ki67 and reovirus RNA) also displayed the strongest PD-L1 expression. Case 5, in contrast, displayed relatively low expression.

![Fig. 3. Tumor immune cell infiltration.](image-url)

(A) Fold change in cell-surface intercellular adhesion molecule (ICAM) expression on CD4 and CD8 T cells from trial patients' peripheral blood. (B) Trial and control patient IHC tumor sections stained for CD3 (brown). Scale bars, 20 μm. "V" indicates blood vessel. (C) Trial and control patient IHC tumor sections stained for CD8 (brown). Scale bars, 20 μm.
of Ki67, reovirus RNA, and PD-L1 (fig. S8C). We sought to confirm these clinical findings in vitro; direct reovirus treatment of the established glioma cell line U87, primary human GBM cells [GBM1 and GBM4 (32)], and cell lines derived from metastatic breast cancer, colon cancer, and melanoma (MCF-7, SW620, and Mel624) significantly increased PD-L1 expression in U87 ($P = 0.0021$), GBM4 ($P = 0.0275$), MCF-7 ($P = 0.0002$), and SW620 cells ($P = 0.0062$), with no significant differences in GBM1 and Mel624 cells (fig. S9A).

We reasoned that reovirus treatment would also promote checkpoint protein expression within tumor-infiltrating immune cell populations. In vitro reovirus treatment of patient-derived mixed HGG cell cultures from control patients outside the trial induced PD-L1

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**Fig. 4. Expression of cleaved caspase 3, PD-L1, and PD-1 in HGGs after reovirus treatment.** (A) Representative trial and control patient HGG sections stained for cleaved caspase 3 (brown) by IHC. Scale bars, 60 μm. (B) Representative trial and control patient HGG sections stained by IHC for programmed death ligand 1 (PD-L1) (brown). Scale bars, 30 μm. (C) Representative (one of three samples tested) flow cytometry for PD-L1 on GBM tumor-infiltrating lymphocytes (TILs) (bottom) or peripheral blood mononuclear cells (PBMCs) (top) derived from the same patient, after stimulation for 48 hours using 1 plaque-forming unit per cell reovirus. PBS, phosphate-buffered saline; Ab, antibody. (D) Representative trial and control patient HGG sections stained by IHC for programmed cell death protein 1 (PD-1) (brown). Scale bars, 30 μm. (E) Representative flow cytometry for PD-L1 on GBM1 cells after stimulation with combinations of purified interferon (IFN)–α/IFN–β/IFN–γ for 24 hours, each at 100 pg/ml. (F) Representative flow cytometry for PD-L1 on GBM1 cells after stimulation with ex vivo HGG-derived conditioned medium (CM) or reovirus-conditioned medium (RCM) for 24 hours (at a concentration of 1:4 of CM to native medium). (G) Representative flow cytometry for PD-L1 on GBM1 cells after stimulation using PBMC-derived CM or RCM for 24 hours (at a concentration of 1:4 of CM to native medium) with blockade of IFN–α/β/γ or equivalent isotype controls.
Reovirus induces PD-L1 expression via an IFN-based mechanism

We used GBM1 cells as an in vitro model of HGG tumor cells to confirm an IFN-dependent mechanism for reovirus-induced stimulation of PD-L1 expression. GBM1 cells were treated using type I IFNs (IFN-α and IFN-β) and type II IFN (IFN-γ) in isolation or in combination. Whereas type I or type II IFN treatments each induced 50 to 70% up-regulation of PD-L1, the combination of type I and type II IFNs induced a 250% increase in cell surface PD-L1 expression. By contrast, combining IFN-α and IFN-β, which bind the same type I IFN receptor, induced no further increase in PD-L1 expression over IFN-α alone (Fig. 4E and fig. S10A). Reovirus treatment of fresh patient-derived HGG single-cell suspensions (including all cell types contributing to the tumor immune microenvironment) generated reovirus-conditioned medium (RCM), which contained high concentrations of type I and II IFNs (fig. S10B). RCM was filtered to remove reovirus, and conditioned medium (CM) controls were also filtered to maintain experimental consistency. Soluble factors within HGG-RCM significantly up-regulated GBM1 PD-L1 expression in comparison to CM (P = 0.00481) (Fig. 4F and fig. S10C). To establish the relative contributions of type I and type II reovirus-induced IFNs in the up-regulation of PD-L1, we used PBMC-RCM (containing IFN-α, IFN-β, and IFN-γ) to treat GBM1 cells with concurrent blockade of IFN receptors and soluble IFNs. Blockade of type I or II IFNs partially reduced PBMC-RCM–induced PD-L1 expression on GBM1 cells, whereas blockade of both type I and II IFNs greatly diminished PD-L1 expression, confirming that type I and II IFNs cooperate to induce PD-L1 in patient-derived glioma cells (Fig. 4G and fig. S10D).

Sequential treatment using intravenous reovirus followed by PD-1/PD-L1 axis blockade improves survival in mice with brain tumors

On the basis of the above data showing immune cell infiltration and up-regulation of the PD-1/PD-L1 axis by systemic reovirus in brain tumor patients, we sought to test survival using sequential OV checkpoint inhibitor blockade in an immunocompetent orthotopic animal model of glioma. Consistent with our trial data, C57/BL6 mice implanted intracranially with GL261 glioma cells exhibited improved survival using intravenous GM-CSF/reovirus [our optimal systemic reovirus regime (22)] over a 2-week period, followed by a 1-week period of PD-1 antibody treatment, compared to treatment with either virotherapy or checkpoint blockade alone (Fig. 5A). Comparison of hematoxylin and eosin–stained sections from brain tumors taken postmortem from GM-CSF/reovirus-treated mice revealed prominent perivascular and intratumoral inflammatory infiltrate, containing lymphocytes (Fig. 5B). Further, flow cytometry analysis of brain tumor single-cell suspensions revealed significantly higher active (IFN-γ+) helper (CD3+CD4+) and cytotoxic (CD3+CD8+) T cells within GM-CSF/reovirus-treated tumors in comparison to PBS-treated tumors (P = 0.0089 and P = 0.0125, respectively, for helper and cytotoxic T cells; Fig. 5C).

DISCUSSION

Our data provide evidence of an OV, reovirus, gaining access to brain tumors after intravenous administration to patients. Reovirus RNA was widely detected in tumor cells of differing histological types. The intravenous route, therefore, holds promise as an efficient means of delivering OV to brain tumors, enabling regular scheduled treatments to be administered, while avoiding the need for neurosurgical methods of access.

The tight window of opportunity between clinical presentation and planned brain surgery limited the number of patients able to participate in this study. Nonetheless, tumors from all nine treated patients, across a range of histological tumor types, showed evidence of reovirus infection. An ongoing clinical trial is assessing the safety and efficacy of intravenous reovirus in combination with postoperative chemoradiotherapy for patients with GBM (ReoGlio, ISRCTN70044565). Other OVs, including parvovirus H-1, are also being tested by intravenous infusion in patients with brain tumors (33).

Previous studies have shown that apoptosis in malignant cells is induced after reovirus receptor binding and disassembly to form intermediate subviral particles but that viral genome transcription and translation are not required (34). Hence, the lack of evidence for major reovirus productive infection in our study does not necessarily mean the absence of direct viral cytoxicity. Our RNA-seq analysis and IHC for cleaved caspase 3 do indicate the induction of apoptosis. Moreover, from an immunotherapy perspective, intracellular reovirus RNA is sufficient to engage pathogen recognition receptors, inducing IFN expression. IFNs are critical mediators of immune-mediated anticancer effects, for example, by activating NK cell, T cell, and dendritic cell populations and enhancing antigen presentation (35). Future studies should, therefore, look to optimize reovirus treatment schedules to further increase the delivery of reovirus to tumor cells, given the absence of major reovirus productive infection.

The BBB is known to be disrupted in brain tumors, as indicated by the presence of vasogenic edema (36). Nonetheless, many systemic anticancer agents, including monoclonal antibodies, are thought to be excluded by the BBB due to their higher molecular mass (37, 38). The mechanisms by which reovirus enters brain tumors in comparison to tumors outside of the brain and its relation to the integrity of the BBB remain unclear, although the association of reovirus with multiple peripheral WBC subsets supports the idea that these immune cells may play a role in the delivery of virus to tumor. This is additionally supported by our observations of enhanced immune cell tumor infiltration after reovirus and also by the clinically observed lymphopenia in all nine treated patients, a phenomenon that could be attributed at least in part to the accumulation of lymphocytes at the site of infection in tumor, consistent with previous reports of lymphopenia in acute viral infections (39). We found up-regulation of CCL3 and CCL4 mRNA in brain tumors from reovirus-treated patients. These chemokines are up-regulated in other acute viral encephalitis infections, including Semliki Forest virus and West Nile virus (40), whereas blockade of CCR5, whose ligands include CCL3 and CCL4 (41), decreases leukocyte migration into murine brains after viral infection (40). Preexisting CD3+ and CD8+ TILs have a positive effect on survival in solid tumors, and infiltrating cytotoxic T cells are critical for PD-1/PD-L1–directed immunotherapy (29, 42). In our trial, a proportion of brain tumors contained high numbers of cytotoxic T cells after reovirus treatment, a finding consistent with our murine data showing increased T cell infiltration into tumors after systemic reovirus treatment. Our data indicate lower baseline CD68 staining than in previous reports (43).
Fig. 5. Combination intravenous reovirus and checkpoint inhibition in an orthotopic syngeneic brain tumor model. C57/BL6 reovirus-vaccinated mice (22) were injected with GL261 cells intracranially on day 1 and treated using combinations of granulocyte-macrophage colony-stimulating factor (GM-CSF) plus intravenous reovirus and/or anti–PD-1 antibody. (A) Kaplan-Meier survival plot, with Mantel-Cox comparison of survival curves: control versus anti–PD-1 (P = 0.4617), control versus GM-CSF/reovirus (P = 0.0012), control versus GM-CSF/reovirus + anti–PD-1 (P < 0.0001), GM-CSF/reovirus versus GM-CSF/reovirus + anti–PD-1 (P < 0.0001), and anti–PD-1 versus GM-CSF/reovirus + anti–PD-1 (P < 0.0001). (B) Representative brain tumor hematoxylin and eosin–stained sections from PBS-treated and GM-CSF/reovirus-treated mice. Black arrows mark vascular endothelial cells; white arrows mark lymphocytes. Scale bars, 30 μm. (C) Flow cytometry quantification of CD3+ CD4+ IFN-γ+ or CD3+ CD8+ IFN-γ+ TILs from PBS-treated or GM-CSF/reovirus-treated mice. Graph shows the mean ± SD of four samples.

potentially due to differences in staining methods. The functional relevance of increased CD68 cells in brain tumors after intravenous reovirus infusion and their origin remain unclear, warranting further investigation.

Tumors from reovirus-treated patients exhibited more intense staining for both PD-1 and PD-L1, immune checkpoint proteins that are induced by IFNs. We further found evidence for both peripheral and tumor induction of IFNs, key cytokines in reovirus-mediated activation of immune cell populations (44). It has previously been shown that effector T cells up-regulate PD-1 during the acute phase of viral infections while maintaining activity and that this protective response does not correspond to adverse outcomes (45, 46). One of the determinants of efficacy in PD-1 checkpoint blockade is tumor expression of PD-L1 (47). In GBM, PD-L1 expression is relatively weak in most of the tumors (48). We found PD-L1 to be strongly up-regulated by type I IFNs in combination with IFN-γ, cytokines that were secreted after ex vivo reovirus treatment of HGG cells. Hence, reovirus therapy may be used to improve clinical outcomes in patients with brain tumors by activating WBCs, enhancing T cell infiltration into tumors, and up-regulating PD-L1 there, in preparation for subsequent anti–PD-1 therapy. In support of our findings, Ribas et al. has reported findings from the phase 1b portion of the MASTERKEY-265 study in extracranial advanced melanoma, where patients were treated using intratumoral injections of T-VEC (HSV type 1 encoding GM-CSF), with concomitant anti–PD-1 therapy beginning 6 weeks after the start of OV therapy (49). Patients who responded to treatment in this study had increased CD8+ T cells and elevated PD-L1 protein expression on several cell subsets in tumors after OV therapy.

The combination of intravenous OV with anti–PD-1 therapy for the treatment of brain tumors is the focus of investigations in our laboratory. In support of our findings, preclinical research using Maraba virus in murine models of triple-negative breast cancer has also shown OV-induced tumor inflammation, immune infiltration, and up-regulation of PD-L1, leading to enhanced survival when Maraba virus is combined with PD-1 checkpoint blockade (50). Rational drug combinations that include anti–PD-1 therapy for the treatment of brain tumors are all the more pertinent in light of the failure of single-agent nivolumab to deliver a survival advantage in patients with GBM (NCT02017717) (51). The challenges of local delivery of OVs into brain tumors are substantial, and direct intratumoral injection is inevitably suboptimal for multifocal/infiltrative disease. Systemic virus-based immunotherapy provides a pragmatic alternative and appears capable of altering the immune microenvironment within brain tumors, which in turn, could potentially improve cancer therapy when combined with immune checkpoint blockade.

MATERIALS AND METHODS

Study design
European Union Drug Regulating Authorities Clinical Trials (EudraCT) number 2011-005635-10. This was an open-label, nonrandomized, single-center study, which recruited nine adult patients between July 2013 and November 2014 at The Leeds Teaching Hospitals National Health Service Trust (LTHT), Leeds, United Kingdom. Patients were planned
for debulking neurosurgery either for recurrent HGG or for metastatic tumor to the brain, as part of routine clinical care. A single 1-hour intravenous infusion of $1 \times 10^{10}$ TCID$_{50}$ reovirus was administered to patients ahead of surgery. The primary end point of the study was the presence of reovirus in the resected tumor sample. Tumors were initially analyzed for the presence of reovirus in batches of three. The trial achieved its primary end point and was closed after the recruitment of six patients with recurrent HGG and three with metastatic tumors to the brain, because reovirus was detected in all nine tumors. Inclusion criteria included adequate hematological and organ function and an Eastern Cooperative Oncology Group performance status of $\leq 1$. All patients gave written informed consent according to good clinical practice guidelines. Protocol, patient information sheet, and consent forms were approved by the United Kingdom Medicines and Healthcare products Regulatory Authority, regional ethics review committee, and institutional review board at St James’s University Hospital. The trial management committee met on a monthly basis to discuss study progress, including patient safety and adverse events. Clinical patient safety assessments were performed within 1 week of start of treatment, on the day of reovirus infusion (day one), day three, the day of surgery, and 1 month after surgery. Imaging was performed as for standard clinical care only. Control brain tumors were obtained from patients undergoing routine planned surgery at LTHHT. Written informed consent was obtained in accordance with local institutional ethics review and approval. After surgery, all brain tumors were transported in 1-glutamine–containing RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Biosera) and 1% (v/v) antibiotic antimycotic solution (Sigma).

**Reovirus**

Clinical grade reovirus Dearing type 3 (REOLYSIN) was provided by Oncolytics Biotech Inc.

**Animal experiments**

In vivo animal models were approved by the University of Leeds Local Ethics Review Committee or the Mayo Foundation Institutional Animal Care and Use Committee. For fig. S1, 6- to 10-week-old C57/BL6 mice (the Jackson Laboratories) were injected intracranially with $1 \times 10^6$ B16 melanoma cells and 8 days later treated with a single injection of $1 \times 10^6$ plaque-forming units (PFU) intravenous reovirus or PBS. Mice were sacrificed 3 days after treatment. For Fig. 5, eight mice in each group were used; 6- to 8-week-old C57/BL6 reovirus-vaccinated mice (22) were injected intracranially with GL261 cells on day one. On day five, mice were treated using daily intravenous injections of 300-ng GM-CSF (PeproTech) and intravenous reovirus at $5 \times 10^6$ PFU or PBS as a control for 5 days. Treatments were repeated for a further five consecutive days starting on day 12. On days 19, 21, and 23, mice were treated with anti–PD1 antibody (clone RMP1-14; Bio X Cell) or immunoglobulin G (IgG) isotype control (clone MPC11; Bio X Cell) by intraperitoneal injections. Mice were regularly monitored for any signs of deterioration or weight loss, upon which animals were sacrificed, and the duration of survival was recorded.

**IHC, ISH, and IF**

These techniques were performed on formalin-fixed paraffin-embedded tissue sampled randomly from one to two areas of resected patient or animal tumor. Tissue for IHC was processed using an automated Bond Max system (Leica Biosystems) as described (52). Reovirus $\alpha$3 and cleaved caspase 3 antibodies were diluted 1:1000. IHC detection of PD-1, PD-L1, CD3, CD8, CD68, and Ki67 used an automated Bond Max system (Leica Biosystems) according to the manufacturer’s protocol. Primary antibodies (all Abcam) were diluted either 1:500 (PD1 and PD-L1) or 1:200 (CD3, CD31, CD8, CD68, and Ki67). The percentage of positive cells was determined using the InForm System (PerkinElmer). ISH for reovirus RNA and IF for reovirus $\alpha$3 protein, reovirus RNA, and tubulin were performed as previously described (21, 52). IF nuclear counterstaining was with 4′,6-diamidino-2-phenylindole diluted 1:10,000 (Thermo Fisher Scientific). One to two slides per tumor were examined for quantification of reovirus $\alpha$3 protein and reovirus RNA, whereas two to three slides per tumor were examined for quantification of Ki67, PD-1, PD-L1, and CD markers.

**Peripheral reovirus carriage RT-PCR**

Peripheral blood samples taken from study patients mid–reovirus infusion were collected, fractionated for PBMCs and granulocytes, and then RNA-extracted as previously described (21). PBMC subsets were isolated by MACS Micro bead selection (Miltenyi Biotec) according to the manufacturer’s instructions. PCR conditions and primers were also previously described (53). Samples were run on 2% agarose gels alongside a 100-bp (base pair) DNA ladder (New England BioLabs).

**Tumor reovirus qRT-PCR**

Tumor tissue was disaggregated using the Cell Dissociation Sieve and Tissue Grinder Kit (Sigma). One microgram of TRIzol (Sigma)–extracted RNA was reverse-transcribed (Bioline SensiFAST cDNA Synthesis Kit) according to the manufacturer’s instructions in 20-μl reactions. Two microliter of resultant reaction containing cDNA was subjected to standard quantitative polymerase chain reaction using SYBR Green PCR Master Mix (Life Technologies) and primers specific to the reovirus $\alpha$3 gene to amplify a 61-bp product (forward, 5′-GATGCCAGATCTCTTAATCA-3′ and reverse, 5′-CTCCTCGCAATACAATCTCGT-3′; both Sigma). Quant iTect primers (Qiagen) specific to 18S rRNA served as cellular RNA controls.

**RNA sequencing**

Three control GBM tumors were compared to three GBM tumors from reovirus-treated trial patients. RNA-seq libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina), and 150-bp paired end reads were sequenced on a lane of the Illumina HiSeq3000. Reads were quality-processed and aligned as described previously (54). Expression analysis was performed using the Cuffdiff suite of programs (55). Briefly, expression was quantified using cuffquant with multiread correction and abundant noninformative transcripts (ribosomal and mitochondrial RNAs) masked. Differential expression analysis between the control (patients without reovirus) and treated (patients receiving reovirus) groups was then performed using Cuffdiff with a false discovery rate of 0.1. Gene set analysis of the resulting significantly differentially expressed genes was performed using hypergeometric testing via WebGestalt (56), with the set of genes expressed in any sample as the reference set.

**Processing of HGG single-cell suspensions and PBMCs**

Written informed consent was obtained in accordance with local institutional ethics review and approval. HGG tumor samples were processed using the Brain Tumor Dissociation Kit (Miltenyi Biotec), according to the manufacturer’s instructions. Briefly, tumor tissue

was homogenized and digested in gentleMACS C tubes using a dissociator and buffers and enzymes supplied by the manufacturer and then incubated at 37°C. Single-cell suspensions were passed through a 70-μm filter. Where appropriate, samples were demethylated using Myelin Removal Beads II (Miltenyi Biotec). Paired PBMC samples were derived from the same patients by step density centrifugation over Lymphoprep (Axis-Shield).

**Culture of GBM1, GBM4, MCF-7, U87, SW620, and Mel624 cell lines**

All cells were cultured at 37°C with 5% CO₂. The human breast adenocarcinoma cell line (MCF-7), human colorectal cancer cell line (SW620), human glioblastoma cell line (U87), and the melanoma cell line (Mel624) were maintained in 1-glutamine-containing Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% (v/v) FCS. GBM1 and GBM4 cells were adherently propagated on plasticware coated with poly-L-ornithine (5 µg/ml; Sigma) and laminin (5 mg/ml; Invitrogen) and cultured in Neurobasal medium supplemented with 0.5× B27, 0.5× N2 (all from Thermo Fisher Scientific), recombinant human basic fibroblast growth factor (40 ng/ml; Gibco), and epidermal growth factor (40 ng/ml; R&D Systems).

**Generation of CM and RCM from fresh patient HGG samples and PBMCs**

HGG single-cell suspensions or PBMCs were cultured for 24 hours at 2 × 10⁶/ml alone (CM), with 50 PFU per cell reovirus (HGG-RCM) or with 1 PFU per cell reovirus (PBMC-RCM) in 1-glutamine-containing RPMI 1640 supplemented with 10% (v/v) FCS. Cell-free supernatants were collected, and reovirus was removed by filtration through Millipore OptiScale 25 filters. CM and RCM were stored at −80°C until required.

**Flow cytometry**

Flow cytometry was performed on an LSRII flow cytometer, and data were analyzed using FACSDiva (both Becton Dickinson) or FlowJo (TreeStar). Relative fluorescence shift (RFS) was calculated using the formula RFS = (MFI of treatment sample − isotype) / (MFI of control sample − isotype), where MFI is median fluorescence intensity. To create overlay plots in FlowJo, we normalized the y axis to mode.

**Direct reovirus stimulation of patient HGG and PBMC**

HGG single-cell suspensions and PBMCs were plated separately at a density of 2 × 10⁶/ml and cultured with or without reovirus at 1 PFU per cell for 48 hours in RPMI 1640 supplemented with 10% FCS. Samples were harvested and washed with isosmotic buffer (PBS/0.05% bovine serum albumin) before staining with the following antibodies: CD45-FTC (fluorescein isothiocyanate) (H130), CD8-BV421 (RPA-T8), CD20-BV395 (2H7), NKP46-APC (9E2/NKp46), and PD1-PE (MIH4) from BD Biosciences; CD3PerCP (BW264/56) and CD4-PEvio770 (M-T466) from Miltenyi Biotec; CD69-BV421 FN50 and PDL1-PE (29E2A3) from BioLegend; mouse CD3-FTC, CD4-PerCP, and CD8-PE from BioLegend; and mouse intracellular IFN-γ-PE-Cy7 from BioLegend.

**Direct reovirus stimulation of primary human cells and cell lines**

GBM1, GBM4, MCF-7, U87, SW620, and Mel624 cells were cultured overnight at 1 × 10⁵ cells per well. Reovirus (0, 1, or 10 PFU per cell) was then added for 24 hours before cells were harvested and stained with anti-human PD-L1–PE (clone MIH1; eBioscience) as above. The statistical analysis was conducted using GraphPad Prism software. Asterisks in Fig. 5C and figs. S2B, S8, and S9 represent *P < 0.05 using a t test. The statistical correlation of reovirus RNA/protein with Ki67 (Fig. 2B) and with time between infusion and surgery (fig. S6) was determined by linear regression.


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Data and materials availability: Correspondence and requests for materials should be addressed to A.S., A.A.M., or S.C.S. Oncolytic reovirus (REOLYSIN) was supplied by Oncolytics Biotech Inc. under a material transfer agreement with the University of Leeds, United Kingdom.

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Intravenous delivery of oncolytic reovirus to brain tumor patients immunologically primes for subsequent checkpoint blockade

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Viruses team up with cancer immunotherapy

Immune checkpoint inhibitors have shown great promise for cancer therapy, but they do not treat all cancers, and neither breast nor brain tumors are usually treatable with these drugs. However, Bourgeois-Daigneault et al. discovered a way to address this for breast cancer, and Samson et al. discovered a way to address this for brain tumors. In both cases, the authors found that oncolytic virus treatment given early, before surgical resection, alters the antitumor immune response and potentiates the effects of subsequent treatment with immune checkpoint inhibitors. Although these studies differ in the details of their methods and the immune effects induced by the oncolytic viruses, they indicate the potential of such viruses for enhancing the potential of checkpoint therapy and expanding it to new types of cancer.